EFFECTS OF NEONATAL DOMOIC ACID TREATMENT AND SOCIAL ISOLATION REARING ON MEASURES OF ATTENTIONAL PROCESSING IN RATS: AN INVESTIGATION OF THE "TWO-HIT" HYPOTHESIS OF PSYCHIATRIC DISEASE

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ABSTRACT

Neuropsychiatric disorders affect a significant number of people across the globe and are a serious economic and social burden. As a class of diseases they are often poorly understood, difficult to treat, and can lead to a significant decline in normal function and quality of life. Recent evidence supports the notion that many neuropsychiatric disorders are neurodevelopmental in foundation, originating at least in part from abnormal activity during critical periods of brain development. Attentional processing is a higher order cognitive behaviour fundamental to many important brain processes and is known to be affected in a number of human neuropsychiatric disorders. The purpose of the research described herein was to better understand how changes in early brain development can have a long lasting effect on the central nervous system relevant to the etiology of neuropsychiatric disorders, and to utilize a novel approach to investigate the "two-hit" hypothesis of psychiatric disease by probing the effects of chemically (neonatal low-dose domoic acid [DOM] treatment) and/or environmentally (social isolation rearing) induced changes in attentional processing.

First, adult untreated rats were used to develop a latent inhibition (LI) paradigm that resulted in both deficient and abnormally persistent LI. Next, utilizing both the newly-developed LI protocol and previously established protocols for prepulse inhibition (PPI), the behavioural consequences of neonatal DOM administration and social isolation housing, both alone and in combination, were measured. Results indicated a number of behavioural alterations in both sexes and across all treatment groups indicating that neonatal DOM treatment and isolation rearing affect both of these measures of attentional processing although not in identical ways. Subsequently, the regional expression profiles of various proteins involved in dopaminergic and GABAergic neurotransmission were quantified by Western blot analysis to investigate the potential molecular alterations responsible for the observed behavioural changes. No significant effects on these markers were observed in any treatment group, although large inter-individual variations were noted. A preliminary post-hoc analysis of correlations between changes in PPI and protein expression resulted in a number of significant correlations and suggested that the various experimental manipulations were differentially affecting the signalling pathways involved.

In conclusion, both neonatal low-dose DOM treatment and social isolation rearing affect the development of attentional processing in rats. However, each paradigm may exert these effects through different neuronal signalling systems and these different systems may be responsible for different aspects of the behavioural changes that were observed. Finally, this work illustrates the potential of a novel "two-hit" method for studying the development of neuropsychiatric disease as well as the complex interconnected processes that are affected by altered brain development.

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DEDICATION

This thesis is lovingly dedicated to my parents
Darrell and Pauline Adams
who taught me to love learning
and who always told me to follow my dreams

to my sister Ashley
my best friend and partner in crime

and to my husband Nathan
whose constant support, encouragement and love
have sustained me through the bad times
and made the good times worthwhile

TABLE OF CONTENTS

Chapter 2 - Development of protocols to assess various aspects of latent inhibition behaviour in adult, untreated rats using a conditioned emotional response task

Chapter 3 - Chemical and environmental effects on behavioural measures of attentional processing

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ACh	acetylcholine
AD	adjusted density
ADHD	attention deficit hyperactivity disorder
AMPA	α–amino-3-hydroxy-5-methyl-4-isoxazolepropionate
BDNF	brain derived neurotrophic factor
Ca^{2+}	calcium ion
CER	conditioned emotional response
Cl^{-}	chloride ion
CNS	central nervous system
COMT	catechol-O-methyl transferase
CS	conditioned stimulus
CTA	conditioned taste aversion
DA	dopamine
dB	decibel
DG	DOM / group housed
DISC1	disrupted in schizophrenia 1
DOM	domoic acid
DS	DOM / single housed
DSM	diagnostic and statistical manual of mental disorders
DTNBP1	dysbindin
EAA	excitatory amino acid
EC	entorhinal cortex
EEG	electroencephalograph

GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GDP	gross domestic product
Glu	glutamate
HB	homogenization buffer
iGluR	ionotropic glutamate receptor
i.p.	intraperitoneal
K^+	potassium ion
KA	kainate
LAI	long-acting injectable
LI	latent inhibition
LTP	long term potentiation
mA	milliamp
mGluR	metabotropic glutamate receptor
mRNA	messenger ribonucleic acid
ms	millisecond
MWM	Morris water maze
Na^+	sodium ion
NAc	nucleus accumbens
NMDA	N-methyl-D-aspartate
NPE	non pre-exposed
NRG1	neuregulin-1
NVH	neonatal ventral hippocampal
OCD	obsessive compulsive disorder

PCP	phencyclidine
PE	pre-exposed
PFC	prefrontal cortex
PND	postnatal day
PPI	prepulse inhibition
PTSD	posttraumatic stress disorder
RD	relative density
RV	raw value
s.c.	subcutaneous
SEM	standard error of the mean
SG	saline / group housed
SLG	Schmajuk-Lam-Gray
SS	saline / single housed
TH	tyrosine hydroxylase
Trk	tyrosine receptor kinase
US	unconditioned stimulus
VTA	ventral tegmental area

Chapter 1

General introduction

1.1 Overview of psychiatric disease

Psychiatric diseases represent a diverse group of disorders and illnesses which can be classified in a variety of ways. The current Diagnostic and Statistical Manual of Mental Disorders (DSM) v.5 includes a number of categories outlined in Table 1.1 (American Psychiatry Association, 2013). Such diseases have a devastating cost to society with regard to the economic impact and the personal cost to those affected and their families.

The cost of treating mental illness was not historically considered a global health priority, with focus generally centered on communicable diseases as well as non-communicable illnesses such as cancer and cardiovascular disease. The focus on mental illness has increased as we have gained a better understanding of the impact and cost of these diseases. The cumulative global effect of mental illness on lost economic output has been estimated to amount to US $16 trillion in the next 20 years, equivalent to 25% of the global gross domestic product (GDP) for 2010 (Bloom *et al*, 2011). Despite the high personal and economic costs, treatment rates for people with mental and substance use disorders are low, particularly in developing countries. Even in developed countries with modern healthcare, treatment is typically provided many years after the disorder first begins (Whiteford *et al*, 2013).

In Canada, mental illness is a significant economic burden with the estimated total cost being as high as $51 billion in 2003 (Lim *et al*, 2008). This includes the cost of both direct government funded health care services as well as the indirect cost of lost productivity due to disability and premature mortality (Stephens and Joubert, 2001).

Table 1.1 Classification of mental disorders according to the DSM v.5

Classification	Example of disorders
Neurodevelopmental disorders	Autism spectrum disorder
	Attention deficit/hyperactivity disorder
Schizophrenia spectrum and related disorders	Schizophrenia
	Schizoaffective disorder
Bipolar and related disorders	Bipolar I disorder
	Bipolar II disorder
Depressive disorders	Major depressive disorder
	Premenstrual dysphoric disorder
Anxiety disorders	Specific phobia
	Generalized anxiety disorder
Obsessive-compulsive and related disorders	Obsessive-compulsive disorder
	Body dysmorphic disorder
Trauma and stressor-related disorders	Reactive attachment disorder
	Posttraumatic stress disorder
Dissociative disorders	Dissociative identity disorder
	Depersonalization/derealization disorder
Somatic symptom and related disorders	Somatic symptom disorder
	Illness anxiety disorder
Feeding and eating disorders	Anorexia nervosa
	Bulimia nervosa
Elimination disorders	Enuresis
	Encopresis
Sleep-wake disorders	Insomnia disorder
	Narcolepsy
Sexual dysfunction	Erectile disorder
	Male hypoactive sexual desire disorder
Gender dysphoria	Gender dysphoria in children/adolescents/adults
Disruptive, impulse-control and conduct disorders	Oppositional defiant disorder
	Antisocial personality disorder
Substance-related and addictive disorders	Alcohol use disorder
	Gambling disorder
Neurocognitive disorders	Neurocognitive disorder due to Alzheimer's disease
	Neurocognitive disorder due to Parkinson's disease
Personality disorders	Paranoid personality disorder
	Narcissistic personality disorder
Paraphillic disorders	Voyeuristic disorder
	Exhibitionist disorder
Other mental disorders	Unspecified mental disorder due to another medical condition
Medication-induced movement disorders and other adverse effects of medication	Tardive dyskinesia
	Antidepressant discontinuation syndrome
Other conditions that may be a focus of clinical attention	Relational problems
	Abuse and neglect

1.2 Schizophrenia and related psychiatric disorders

One such widespread psychiatric illness that leads to great financial and personal cost is schizophrenia. This complex and debilitating mental disorder is characterized by impairments in the perception of reality and is found in approximately 1% of the general population, resulting in great emotional cost to those directly affected, as well as large financial cost to the economy worldwide (Knapp *et al*, 2004; Rössler *et al*, 2005). The estimated annual cost of healthcare for those afflicted in Canada alone is over 2 billion dollars. When combined with the added cost of increased unemployment, lost productivity and early mortality, the cost is estimated to be 6.8 billion dollars (Goeree *et al*, 2005).

Believed to arise due to a combination of genetic susceptibility and environmental influence (Rapoport *et al*, 2005), schizophrenia manifests great variability in symptom profiles, developmental timecourse and response to treatment (Tamminga and Holcomb, 2005). While past research has identified a number of genetic linkages, developmental risk factors (see section 1.2.3) and neurobiological elements (see sections 1.2.4 and 1.2.5), because of the highly complex and heterogeneous nature of the disorder, there is still much that is unknown about schizophrenia and as a result, current treatments are far from adequate (see section 1.2.6).

1.2.1 Clinical characteristics and symptoms

While the term schizophrenia was first introduced by Eugen Bleuler in 1911, it is believed that the disorder has been present in all cultures for centuries (Tamminga and Holcomb, 2005). The DSM v.5 identifies a category of disorders known as schizophrenia spectrum and related disorders. This category includes schizophrenia,

schizotypal personality disorder, and other psychotic disorders that are characterized by the key features that define this category and make up the diagnostic criteria. The specific diagnostic criteria for schizophrenia are as follows: Two or more of the following symptoms must be each present for a significant portion of the time during a 1-month period (delusions, hallucinations, disorganized speech, grossly disorganized or catatonic behaviour, negative symptoms). Additionally, at least one of the first 3 stated symptoms must be present. Further, diagnostic criteria state that the level of functioning (occupational, interpersonal, self-care) must be compromised for a significant portion of the time, symptoms must persist for a minimum of 6 months, schizoaffective disorder, depressive disorder or bipolar disorder with psychotic features have been ruled out, and the disturbance cannot be attributed to the physiological effects of a substance or another medical condition. Finally, if there is a history of autism spectrum disorder or a childhood-onset communication disorder, a diagnosis of schizophrenia can only be made in the presence of prominent hallucinations or delusions that are present for at least 1 month, in addition to other potential symptoms (American Psychiatry Association, 2013).

The diagnostic criteria for schizophrenia focus on a very specific set of symptoms. There are, however, a wide variety of symptoms outside of those used for diagnosis. This variety of symptom may be experienced in a multitude of combination, forms and intensities by people within the clinical population. Many of these symptoms, though reliably observed in people with schizophrenia, are also found in other disorders (Baribeau and Anagnostou, 2013; Sugranyes *et al*, 2011; Vöhringer *et al*, 2013). Furthermore, some characteristics, such as prepulse inhibition (PPI) deficits, cannot be detected without specific testing (see section 1.3.4.2), but are believed to be indicative of

serious brain dysfunction that can manifest itself as other, more readily observed symptoms (Braff and Geyer, 1990; Swerdlow and Geyer, 1998).

Thus in practice, the symptoms of schizophrenia are generally classified into 3 categories of positive, negative and cognitive symptoms (Khan *et al*, 2013; Tandon *et al*, 2009). The positive symptoms of schizophrenia refer to symptoms that are present in the clinical population that are not seen in unaffected individuals. These symptoms include hallucinations, delusions, psychomotor agitation and disordered thought (Strauss *et al*, 1974; Tandon *et al*, 2013). Hallucinations are sensory perceptions that are not present in reality. These perceptions can take on many forms and affect any of the senses. While visual, tactile and olfactory hallucinations can occur, auditory hallucinations (such as hearing voices) are the most common (Lewandowski *et al*, 2009). Delusions are false beliefs that are strongly and consistently maintained, even in the face of obvious evidence to the contrary (Appelbaum *et al*, 1999; Iyassu *et al*, 2013). A common example found in people with schizophrenia are delusions of grandeur, which occur when a person believes that they have special powers or knowledge that others do not. Delusions of persecution or control are also common, with the person believing that they are being plotted against or that others are trying to control them through various means (Andreasen and Olsen, 1982; Engh *et al*, 2010). Psychomotor agitation is observed in a subset of people with schizophrenia and presents as hyperactivity or increased stereotypic movement (Powell and Miyakawa, 2006). Lastly, disordered thought is perhaps one of the most common symptoms of this disease and is characterized by disorganized and often irrational thinking (Andreasen and Olsen, 1982).

The negative symptoms of schizophrenia refer to symptoms characterized by a lack of normal behaviours. Such symptoms include blunted affect, anhedonia, alogia and

social withdrawal (Buchanan, 2007; Khan *et al*, 2013; Strauss *et al*, 1974). Blunted affect refers to a lack of emotional reactivity and a decreased range of emotions. This symptom is likely related to anhedonia, another common occurrence in schizophrenia whereby the person experiences a decrease or inability to experience happiness or pleasure. Alogia is poverty of speech, where a person's speech lacks the level of content seen in normal conversation, such as giving only one word answers in response to questions. Finally, social withdrawal is a negative symptom likely related to those mentioned above. If a person is unable to interact normally with others, to experience emotional intimacy, or to derive pleasure from those interactions, they are more likely to isolate themselves socially (Andreasen and Olsen, 1982; Buchanan, 2007; Erhart *et al*, 2006; Kane, 2013).

While schizophrenia symptoms were originally classified as only positive and negative symptom types, cognitive symptoms are now considered to be a core abnormality in the disorder (Aas *et al*, 2014). These symptoms include deficits in learning, memory and executive functioning, as well as attentional deficits, a topic which is central to this thesis (Gold and Harvey, 1993; Lin *et al*, 2014; Schmidt-Hansen and LePelley, 2012). The term executive function is used to indicate the higher order cognitive functions mediated largely in the prefrontal cortex (PFC) including problem solving, planning, initiation, hypothesis generation, cognitive flexibility, decision making, abstract thinking and judgment (Aas *et al*, 2014). Attentional deficits are also commonly found. Attentional processing is a complex process that allows a person to filter relevant and irrelevant information, hold and manipulate that information, and monitor responses to stimuli (see section 1.3) (Strauss *et al*, 2006).

Although not considered sufficient to meet diagnostic criteria alone, there are a number of other characteristics often found within the schizophrenia population including altered responses to novelty (Cortiñas *et al*, 2008) and an altered sensitivity to nicotine (Brown *et al*, 2012; Mobascher and Winterer, 2008).

1.2.2 Subtypes and comorbidities

As detailed above, there are a large variety of symptoms and characteristics observed in the schizophrenia population, with many people showing vastly different symptom profiles although they have been given the same clinical diagnosis. Symptoms can also change dramatically over time, even within the same person. This clinical heterogeny has lead to the suggestion that perhaps what we currently define as schizophrenia is actually a combination of disorders that often overlap in clinical presentation (Kirkpatrick *et al*, 2001). Additionally, there are a variety of specifications for schizophrenia outlined by the DSM including first episode vs. multiple episodes, current state (acute, partial remission, full remission), and with the presence of catatonia (American Psychiatry Association, 2013).

While the negative and cognitive symptoms are reliably found in people with schizophrenia, they are not solely seen in schizophrenia and may be present in other neurological disorders. This finding, combined with the fact that positive and negative symptoms have been found to respond differently to drug treatment, suggests that the different groups of symptoms may be the result of different neural abnormalities (Rosenzweig *et al*, 2005). Additionally, similarities in symptom profiles, genetic links and risk factors have linked schizophrenia to other disorders such as autism spectrum

disorder (Baribeau and Anagnostou, 2013; Sugranyes *et al*, 2011) and bipolar disorder (Vöhringer *et al*, 2013).

Comorbidity between schizophrenia and other disorders is common. People with schizophrenia are at an increased risk for substance abuse and addiction (Regier *et al*, 1990). Psychiatric comorbidities are observed among people with schizophrenia and occur in a significant portion of patients. While numbers can vary widely, a study by Buckley *et al*, (2009) which compiled a variety of previous results into weighted averages stated that anxiety disorders are one common area of comorbidity with estimated prevalence including 15% for panic disorder, 29% for posttraumatic stress disorder (PTSD) and 23% for obsessive compulsive disorder (OCD). Perhaps the most common comorbidity is depression with an estimated 50% of people with schizophrenia also suffering depression (Buckley *et al*, 2009). Moreover, schizophrenia also presents with non-psychiatric disease states. There is a frequent comorbidity between epilepsy and schizophrenia with the disease occurring more often in people with epilepsy than within the general population (Clarke *et al*, 2012; Kandratavicius *et al*, 2012; Weber *et al*, 2009). Indeed, it has been found that as many as 32% of people with epilepsy display some type of psychiatric comorbidity (Karouni *et al*, 2010) and some medications are effective against symptoms of both epilepsy and schizophrenia (Liang *et al*, 2010).

Some disorders do not necessarily show extensive comorbidity with schizophrenia or a similar symptom profile, but may still be associated with similar deficits. Such is the case with attention deficit hyperactivity disorder (ADHD) which, like schizophrenia, is associated with deficits in working memory, inhibition and attention (Ross *et al*, 2000). Furthermore, ADHD-like features are more prevalent in

young relatives of people with schizophrenia than in the general population, indicating a potential further link between these two disorders (Keshavan *et al*, 2003).

1.2.3 Neurodevelopmental origins of psychiatric disease

Although the cause of schizophrenia is not yet known, the prominent theory is that it is neurodevelopmental in origin and arises due to a combination of genetic susceptibility and environmental factors. According to this theory, events which occur long before the formal onset of the illness (potentially during gestation and/or early life), disrupt the normal development of the central nervous system (CNS) leading to significant and long-lasting changes in CNS functioning (Rapoport *et al*, 2005).

Although schizophrenia can, and does, occur in people who have no family history of the disorder, the risk increases dramatically with the degree of genetic closeness to a person who is afflicted (Tamminga and Holcomb, 2005). The search for this genetic link to has resulted in a number of genes being associated with schizophrenia including neuregulin-1 (NRG1) (Stefansson *et al*, 2002), dysbindin (DTNBP1) (Straub *et al*, 2002), catechol-O-methyl transferase (COMT) (Egan *et al*, 2001) and Disrupted in Schizophrenia 1 (DISC1) (Millar *et al*, 2000). While all of these candidate genes have been linked in some way with schizophrenia, the search for a concrete genetic link to the disorder has thus far been unsuccessful. The search for a genetic link is further compounded by the fact that, given the pattern of inheritance, schizophrenia likely results from multiple genes of susceptibility working in combination with environmental factors (Tamminga and Holcomb, 2005).

While genetic factors likely contribute to the development of schizophrenia by causing an individual to be more vulnerable to the illness, a variety of early life events

have been implicated in a higher than average risk of developing schizophrenia. These events include maternal illness during gestation (Mednick *et al*, 1988), obstetric complications (Cannon *et al*, 2002; Geddes *et al*, 1999) and toxin exposure (Fiore *et al*, 2004). It is believed that such events can lead to subtle alterations in the functioning of the CNS which may result in an increased vulnerability to environmental triggers later in life (Lieberman *et al*, 2001).

1.2.4 Neuroanatomy of schizophrenia and related disorders

A large variety of neuroanatomical alterations have been observed through post-mortem examination of the brains of people with schizophrenia, with the hippocampus being one area where changes are often seen, (see Table 1.2 for a list of relevant brain areas and their primary functions). Changes include a decrease in cell count and disorganization of the hippocampal pyramidal neurons (Jönsson *et al*, 1999), as well as an overall reduction in hippocampal volume (Narr *et al*, 2005). Enlargement of the ventricles and a decrease in cortical volume are also commonly found (DeLisi *et al*, 1991). Other neuroanatomical changes include but are not limited to reduced expression of tyrosine receptor kinase (Trk) B receptors in the hippocampus and PFC (Takahashi *et al*, 2000), reduced thickness of the corpus callosum (Bachmann *et al*, 2003), elevated levels of brain derived neurotrophic factor (BDNF) in the hippocampus and anterior cingulate cortex (Takahashi *et al*, 2000), reduced gray matter volume in the anterior cingulate cortex and throughout the brain (Takayanagi *et al*, 2013) and a decrease in the density of mossy fibre terminal synapses (Kolomeets *et al*, 2007). The neuroanatomical changes found in people with schizophrenia are as varied as the symptom profiles, with some changes being found only in a subset of the clinical population and/or at a certain

Table 1.2 Relevant brain areas and their primary functions (Carlson, 2007; Rosenzweig *et al*, 2005).

Brain area	Primary function(s)
Amygdala	Part of the limbic system. Involved in the processing of emotions.
Corpus callosum	A large bundle of axons that connects the corresponding regions of the cortex in each hemisphere. Facilitates interhemisphere communication.
Cortex (cerebral cortex)	The outermost structure of the brain. Consists of sensory, motor and association areas and is involved in a variety of processes including memory, attention, creativity, perceptual awareness, judgement, thought, language and consciousness.
Entorhinal cortex	Primary hub between the hippocampus and the cortex
Hippocampus	Part of the limbic system. Involved in learning, memory and spatial navigation.
Hypothalamus	Involved in the regulation of the autonomic nervous system and control of the pituitary gland.
Nucleus accumbens (NAc)	Part of the basal ganglia. Consists of a shell and a core. Receives dopamine innervations from the VTA. Implicated in reinforcement and reward.
Prefrontal cortex (PFC)	The anterior most region of the frontal lobe. Involved in many aspects of cognition, personality and executive functioning.
Striatum	Part of the basal ganglia. Made up of the caudate nucleus and putamen. Receives input from the cortex and transmits to the basal ganglia.
Substantia nigra	Part of the basal ganglia. Plays a role in reward, addiction and movement.
Thalamus	Part of the limbic system. Projects and receives sensory and movement information to/from the cortex
Ventral tegmental area (VTA)	Part of the basal ganglia. Where the mesocorticolimbic system projections originate. Involved in reinforcement and reward.

timepoint during disease progression. While this makes the specific attribution of symptoms difficult, studies attempting to link specific neurological changes to specific disease characteristics are ongoing (Brambilla *et al*, 2013; Palaniyappan *et al*, 2012; Takayanagi *et al*, 2013).

1.2.5 Neurochemistry of schizophrenia and related disorders

Although the cause of schizophrenia is not yet known, current research suggests that alterations in several neurotransmitter systems, notably the dopaminergic and glutamatergic systems, are likely involved in the profile of symptoms that have been described.

1.2.5.1 The dopamine hypothesis

Dopamine (DA) was the first neurotransmitter to be identified as playing a role in the symptoms of schizophrenia, with the DA hypothesis of schizophrenia stating that the positive symptoms of the disorder are caused by the hyperfunction of the DA system (Carlsson and Lindqvist, 1963). The reasoning for this early attention towards DA is based on two findings: Firstly, it was discovered that high doses of the DA agonist amphetamine produce psychotic-like symptoms similar to those seen in schizophrenia, in people unaffected by the disorder. Amphetamine will also exacerbate such symptoms in a person already experiencing schizophrenia induced psychosis (Lieberman *et al*, 1987). Secondly, it was found that many of the drugs that were successful in treating the symptoms of schizophrenia were drugs that acted by blocking DA receptors (Matthysse, 1973). A variety of drugs which act on the DA system have subsequently been used to treat schizophrenia including the older typical antipsychotics which were more effective

at blocking D2 receptors (Seeman and Lee, 1975) and newer atypical antipsychotics which have a greater affinity for the D3/4 receptors (Jardemark *et al*, 2002).

In more recent years, our understanding of the role of DA dysfunction in schizophrenia has become increasingly complex. The DA theory of schizophrenia was reformulated to take into account findings of an overall imbalance in DA across the brain with hyperactivity in the subcortical regions and hypoactivity in the cortical regions (Davis *et al*, 1991; Knable and Weinberger, 1997). It was also suggested that this combined hypofrontality with hyperactivity in the nucleus accumbens (NAc) may be due to the involvement of the excitatory neurotransmitter glutamate (Glu) (Glahn *et al*, 2005).

More recent evidence now points to the involvement of the nigrostriatal pathway as a predominant source of DA dysregulation (Kuepper *et al*, 2012). Furthermore, evidence from translational research including studies in mice with a developmental overexpression of D2 receptors (Kellendonk *et al*, 2006) and studies of people declared high risk for the future development of schizophrenia (Howes *et al*, 2009), have suggested that excess DA may be present in the prodromal phase of disease development and therefore be an early pathological condition leading to irreversible brain dysfunction. See section 1.3.3.1 for more on DA receptors and pathways.

1.2.5.2 The glutamate hypothesis

While the early theories of neurological dysfunction in schizophrenia centered on the DA system, it is now know that other systems are implicated in the pathogenesis of the disorder. Altered functioning of the Glu system has been implicated in symptoms of schizophrenia, with the Glu hypothesis being proposed following the observation that

the administration of N-methyl-D-aspartate (NMDA) receptor antagonists produced schizophrenia-like symptoms in healthy people (Krystal *et al*, 1994; Malhotra *et al*, 1996; Newcomer *et al*, 1999). In addition, administration of NMDA receptors antagonists worsens positive and cognitive symptoms in people with schizophrenia (Lahti *et al*, 1995; Malhotra *et al*, 1997).

According to the Glu hypothesis, some symptoms of schizophrenia may be the result of Glu hypofunction, particularly in the PFC (Olney and Farber, 1995). Interconnections between DA and Glu are common in many areas of the CNS such as the mesocorticolimbic system (Laruelle *et al*, 2005), the hippocampus (Lisman and Otmakhova, 2001) and between glutamatergic afferents and subcortical dopaminergic nuclei (Lisman and Grace, 2005). It is therefore possible, and even likely, that the neuropathology of schizophrenia is the result of an altered interaction between both Glu and DA systems, with one of the most common theories being that hypofunction of the Glu system (particularly within the PFC) results in hyperactivity of the mesolimbic DA neurons (Coyle, 2006). It is important to keep in mind however, that other neurotransmitters have been implicated in these pathways, with γ-aminobutyric acid (GABA) in particular, being thought to play an important role.

Investigation into the involvement of a variety of neurotransmitter systems in schizophrenia is essential, not only to learn more about this disorder, but to pursue potential new therapeutic targets that might impact the large variety of symptoms (Merritt *et al*, 2013). Using the Glu systems as a therapeutic target is also an area of great interest because the commonly prescribed DA antipsychotics have little to no effect on the negative and cognitive symptoms of schizophrenia, which are important

factors of successful social and functional treatment outcomes (Javitt, 1999; Ventura *et al*, 2009).

1.2.6 Current therapies

Therapies for schizophrenia focus on eliminating the symptoms of the disease, with antipsychotic medication being the cornerstone and first-line of treatment. Although a variety of medications are currently available to treat schizophrenia and new ones continue to be developed, issues of tolerability, effectiveness, safety and patient compliance are predominant.

Both older "typical" antipsychotics and newer "atypical" antipsychotics have extensive and serious side effect profiles including movement disorders, significant weight gain, sedation, sexual dysfunction and cardiac issues (Leucht *et al*, 2013). These side effects, combined with difficulty in finding the optimal medication and dose for a given person often leads a patient to stop treatment, with an estimated 84% choosing to discontinue their medication at some point (Kreyenbuhl *et al*, 2011).

Long-acting injectable (LAI) antipsychotic treatment is one option for dealing with patient non-compliance. However, because of issues with medication intolerance and symptom stabilization, LAI is recommended for use only in a sub-population of people, generally those with a history of non-compliance, frequent relapses or who are determined to pose a risk to others. Additionally, LAI may be used in cases where a person demonstrates a low level of insight about their illness and their need for treatment, or if the LAI is requested by the person (Llorca *et al*, 2013).

One novel research path attempting to improve the outlook for those with schizophrenia is the use of pharmacogenetics to predict the response of patients to

various medications. While studies have identified some genomic variants of candidate genes that may be important in the way a person responds to treatment, this research is still in the early stages and not ready for clinical implementation (Tsermpini *et al*, 2014).

The above discussion has focused solely on treatment of the positive symptoms of schizophrenia because they produce the more dramatic occurrences that often lead to a person seeking treatment, and generally must be stabilized with medication before other symptoms can be addressed. Currently used antipsychotics are largely ineffective for the treatment of negative and cognitive symptom. However, the negative and cognitive symptoms of schizophrenia are also debilitating and must be addressed in order for a person to have a good disease outlook (Erhart *et al*, 2006). Current research is focusing on modulation of different targets (e.g. NMDA receptors) with the potential of producing treatment for the negative and cognitive symptoms of schizophrenia, as well as producing drugs that are safer and better tolerated (see Cioffi, 2013 for review).

Other non-pharmacological forms of therapy such as cognitive behavioural therapy have also shown some success but are generally only available once psychotic symptoms have stabilized (Jauhar *et al*, 2014). It is possible that some combination of medication(s) and therapy will be beneficial to some patients (Lin *et al*, 2014).

While the outlook for those people affected by schizophrenia has improved somewhat, a diagnosis of this disease is still likely to result in substantial disability and potentially, in reduced life expectancy due to suicide (Caldwell and Gottesman, 1990; Radomsky *et al*, 1999). For this reason, further research is desperately needed not only to discover and test new treatments, but to learn more about what causes this devastating disease. For the foreseeable future, much of this research will be conducted in pre-clinical animal models. The research described in this thesis will utilize two such animal

models, both alone and in combination. These and other current rodent models of schizophrenia are discussed in detail in section 1.5. It is, however, first necessary to describe two bodies of literature that are fundamental to the work in this thesis, namely attentional processing and the neurotoxin domoic acid (DOM).

1.3 Attentional processing

The brain has inherent limitations to the amount of information that can be processed at any given time (DeFockert, 2013). While attending to incoming information can be accomplished in both overt (shifting gaze to bring something of interest into view) and covert (choosing to listen to one voice over others in a group conversation) manners, in order for any person or animal to function effectively in everyday life it is necessary to filter that incoming information and select which is relevant for further processing (Kinchla, 1992). Attentional processing and information processing are the mechanisms that allow the brain to attend to and process the myriad of incoming information that we deal with on a day-to-day basis. In its simplest form, "attention" can be considered the gateway to the brain (Cohen, 1993).

Attention is not a single phenomenon but is a term used to refer to a variety of complex processes that operates at different levels of awareness. These include the ability to sustain attention over time, the ability to shift attention, the ability to attend to a variety of incoming information, or the ability to attend selectively to a subset of environmental information while filtering out extraneous stimuli (Bushnell and Strupp, 2008). Attention can also be classified as deliberate/controlled or passive/reflexive (Johnston and Dark, 1986). Thus, while we are conscious of much of the attentional processing that occurs, other information is believed to be processed "pre-attentively"

meaning that it is processed automatically, without conscious attention (Kinchla, 1992). This pre-attentive processing generally occurs within the first 100 ms after a stimulus is presented (Ellenbroek, 2004). Prepulse inhibition is one way to measure this automatic form of attentional processing (see section 1.3.4.2)

1.3.1 Attentional processing in mental illness

Attentional processing is a complex process made up of many components including alertness/arousal, focused attention, selective attention, divided attention and sustained attention/vigilance. Attentional deficits can arise from improper functioning in any one or in a combination of these processes (Strauss *et al*, 2006). Changes in the way the brain regulates attention and processes information have been observed in a number of disorders including Alzheimer's disease (Parasuraman *et al*, 1992), Parkinson's disease (Wright *et al*, 1990), bipolar disorder (Burdick *et al*, 2009), and schizophrenia (Nuechterlein and Dawson, 1984), as well as due to ageing (Davies *et al*, 1992).

Deficits in attention have also long been identified as a core feature in schizophrenia and related neuropsychiatric disorders (Anscombe, 1987; Braff, 1993; Cornblatt *et al*, 1985; Nuechterlein and Dawson, 1984). Deficits in attention are present throughout the course of the illness in schizophrenia, can be found before the formal onset of the illness and can persist through treatment (Cornblatt and Keilp, 1994). Additionally, impaired attention can often be observed in the relatives of people with schizophrenia and has been considered as one of the more robust markers of the eventual emergence of the illness within those relatives (Erlenmeyer-Kimling *et al*, 2000; Parnas *et al*, 1982).

While the positive symptoms of schizophrenia are generally considered to be the most dramatic, often garner the most attention, and form the basis for much of the diagnostic criteria (see section 1.2.1) as well as therapeutic intervention (see section 1.2.6) they may not be in fact core to the disorder. It has been suggested that a failure in the ability to reduce the processing of irrelevant incoming information leads to such information being afforded undue attention, resulting in the development of positive symptoms. It has therefore been hypothesized that the positive symptoms of schizophrenia may actually be a consequence of the impairments recognized as cognitive symptoms such as disrupted LI and PPI (Schmidt-Hansen and LePelley, 2012).

1.3.2 Brain areas relevant to attentional processing

A variety of cortical and subcortical areas have been implicated in attentional processing with the specific brain areas, pathways and neurotransmitters systems involved being dependent on which aspect of attentional processing is being considered (Hopfinger *et al*, 2000). Specific brain areas known to be involved in attentional processing that are relevant to the work in this thesis include the PFC, the hippocampus, the NAc, the ventral tegmental area (VTA), the amygdala and the entorhinal cortex (EC) (Cohen, 1993). See Table 1.2 for a brief description of the main role(s) of each area within the brain and sections 1.3.4.1 and 1.3.4.2 for more on the postulated role of these areas in the measures of attentional processing used in this thesis.

1.3.3 Neurotransmission and attentional processing

A number of neurotransmitter systems have been implicated in attentional processing (Agnoli *et al*, 2013; Evans and Drobes, 2009; Klinkenberg *et al*, 2011; Meck

and Williams, 2003; Pehrson *et al*, 2013; Sarter *et al*, 2003) including the DA, Glu and GABA systems.

1.3.3.1 Dopamine

Dopamine is a catecholamine neurotransmitter, a group which belongs to the larger classification of monoamines (Beaulieu and Gainetdinov, 2011). Widely distributed throughout the brain, DA plays important modulatory roles and has been implicated in a diverse array of processes including motor control, neuroendocrine function, cardiovascular control, cognition, emotion and reward (Dziedzicka-Wasylewska, 2004).

In the mammalian brain, four major DA pathways have been identified; the nigrostriatal, tuberoinfundibular, mesocortical and mesolimbic pathways (Prakash and Wurst, 2006). The nigrostriatal pathway projects from the substantia nigra to the dorsal striatum and is an essential component of the extrapyramidal motor system (Prakash and Wurst, 2006). The tuberoinfundibular/ tuberohypophysial pathway originates in the periventricular and arcuate hypothalamic nuclei and projects to the median eminence of the hypothalamus, as well as to the intermediate and posterior lobes of the pituitary gland (Beaulieu and Gainetdinov, 2011). The mesocorticolimbic pathway consists of the mesolimbic and mesocortical pathways respectively. The mesocortical DA pathway originates in the VTA but extends to the frontal cingulate cortex and the EC. The mesolimbic pathway also arises in the VTA, extending to the ventral striatum and parts of the limbic system. It is thought to be involved in motivated behaviour and reward. These projections, illustrated in Figure 1.1, are thought to play a role in emotionality, motivation and cognitive functions (LeMoal and Simon, 1991). Taken together, these

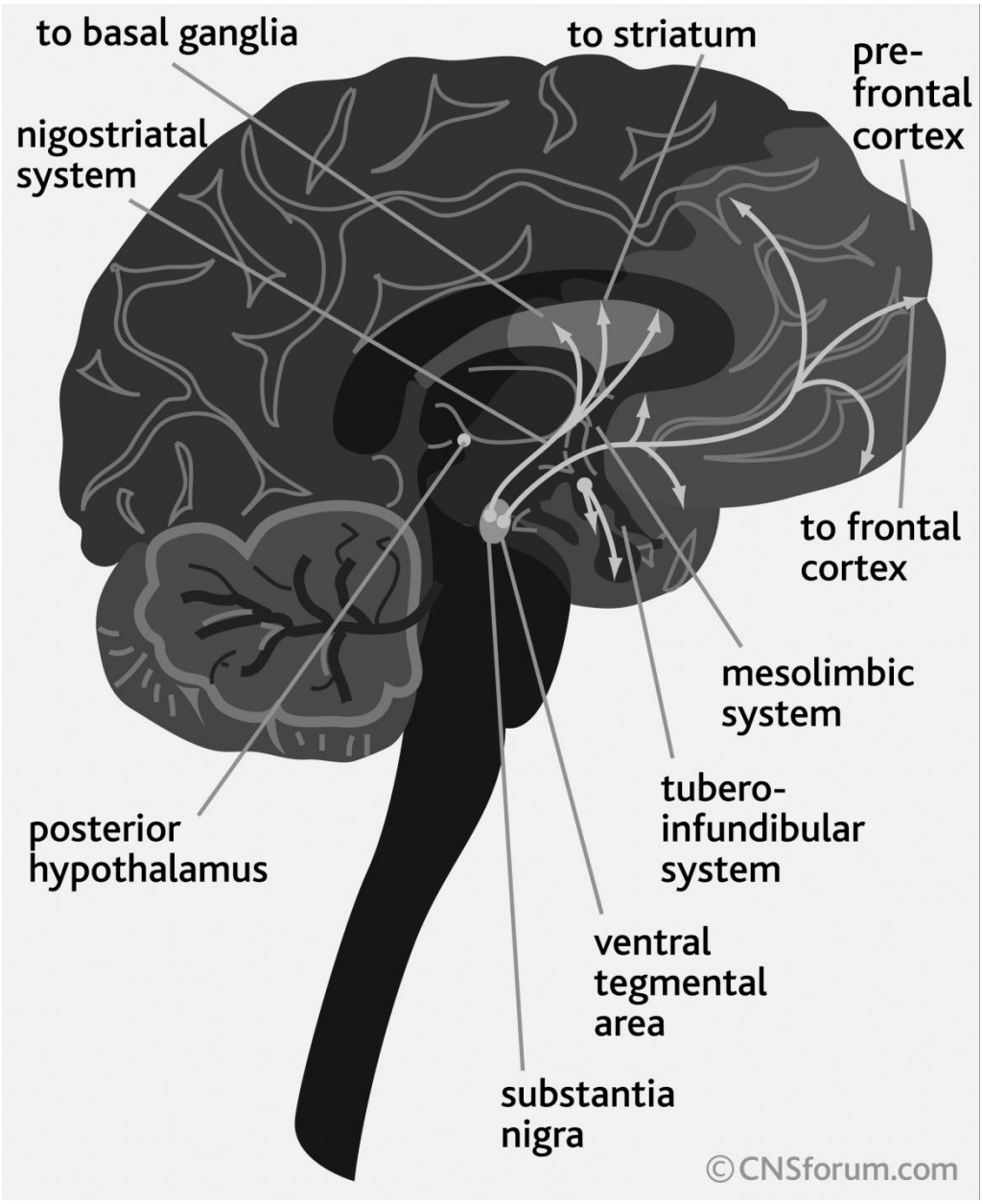

Figure 1.1 A midsagittal diagram of the human brain illustrating the major DA pathways. Figure reprinted with permission from CNSforum.com.

pathways are often referred to as the reward pathway and play an important role in mediating reward, including the rewarding effects of drugs. Accordingly, this pathway is often implicated in drug addiction (Dziedzicka-Wasylewska, 2004).

The physiological actions of DA are mediated by the 5 types of metabotropic, G-protein coupled receptors that are divided into two broad sub-families: D1-like and D2-like receptors (Andersen *et al*, 1990; Beaulieu and Gainetdinov, 2011; Kebabian and Calne, 1979). The D1-like receptor group which includes D1 and D5 receptor subtypes, are coupled with adenylyl cyclase and are excitatory in nature (Tohyama and Takatsuji, 1998), while the D2-like subtype includes D2, D3 and D4 receptors, are often also coupled to adenylyl cyclase but are inhibitory in nature (Tohyama and Takatsuji, 1998). As with other monoamine neurotransmitters, DA generally acts on neuronal circuits via relatively slow modulation of the fast neurotransmission that is mediated by Glu and GABA (Beaulieu and Gainetdinov, 2011).

Because of the importance of DA in regulating many aspects of brain functioning and the widespread network of DA projections, it is unsurprising that a number of severe mental disorders have been associated with the dysfunction or degradation of the DA system. For instance, Parkinson's disease is believed to be caused by the degeneration of DA neurons in the substantia nigra and the subsequent loss of DA in the striatum (Hornykiewicz, 1998; Prakash and Wurst, 2006). Dopamine dysregulation in the mesocorticolimbic system has been linked to a number of problems including drug addiction (Kelley and Berridge, 2002), depression (D'Aquila *et al*, 2000) and schizophrenia (Sesack and Carr, 2002). Dysfunction in of the DA system has also been implicated in a variety of aspects of attentional processing (Nieoullon and Coquerel,

2003) with particular focus devoted to the role of the DA system in LI behaviour (see section 1.3.4.1 for more) (Young *et al*, 2005).

1.3.3.2 Glutamate

Glutamate is the principal excitatory neurotransmitter in the mammalian CNS (Ozawa *et al*, 1998). Glutamatergic pathways and synapses are found throughout the brain and nearly all neurons can be excited by Glu (Curtis and Johnston, 1974). Due to this widespread involvement in many aspects of CNS function, it is no surprise that proper functioning of the Glu system is of critical importance. This is particularly true during development where excitatory amino acids (EAAs) like Glu are involved in a variety of processes including synaptogenesis, dendritic and axonal structure, neuronal survival and synaptic plasticity. During such times of rapid brain growth and change, even small alterations can have significant and long lasting effects, both structurally and functionally. While underactivity can be detrimental, resulting in development being delayed or even permanently disrupted, overactivity can be equally as dangerous, resulting in neuronal alteration and cell death (McDonald and Johnston, 1990).

Glutamate receptors are broadly divided into both ionotropic (iGluR) and metabotropic (mGluR) types. Ionotropic Glu receptors are further divided into three distinct types, NMDA receptors, α–amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors and kainate (KA) receptors. Ionotropic receptors are cation-specific ion channels composed of 4 large subunits forming a central ion pore. At these receptors, the binding of Glu (or an analogue) results in a conformational change to the receptor, allowing sodium (Na^+), potassium (K^+) and/or calcium (Ca^{2+}) ions to pass through the pore (Ozawa *et al*, 1998; Simeone *et al*, 2004; Traynelis *et al*, 2010). The

metabotropic receptors are linked through G-proteins to second messenger molecules and are further divided into three groups based on structure and function. Group I consists of mGluR1 and mGluR5 receptors, Group II consists of mGluR2 and mGluR3 receptors and Groups III consists of mGluR4, 6, 7 and 8 receptors. Group I receptors are located primarily post-synaptically and activate phospholipase C, thereby producing diacylglycerol and inositol triphosphate as second messengers. Receptors from Group II and III are found primarily pre-synaptically and are negatively coupled to adenylyl cyclase. These intracellular messengers can regulate a variety of processes within the cell, including the modulation of voltage-gated and ligand-gated ion channels, as well as gene transcription (Meldrum, 2000; Ozawa *et al*, 1998; Simeone *et al*, 2004).

In addition to being involved in normal functions such as synaptic plasticity, altered Glu functioning has been found to play a role in many diseases and disorders such as Huntington's disease, Parkinson's disease, seizure disorders, Alzheimer's disease, depression and schizophrenia (Bettler and Mulle, 1995; Niciu *et al*, 2013; Olney and Farber, 1995). It is perhaps unsurprising then, that given the symptom profiles of some of these disorders, the Glu system has been found to play an important role in many aspects of attentional processing. Among a host of other observed changes, administration of NMDA receptor antagonists has been found to produce impairments in attention in healthy human subjects (Krystal *et al*, 1994) and animals (Dai and Carey, 1994). A more recent study by Pehrson *et al*, (2013) found that systemic administration of an NMDA receptor antagonist impaired performance on attention tasks in a dose-dependent manner, indicating that these receptors are critical for at least some aspects of proper attentional processing. The Glu systems can also influence attentional processing via its effect on other neurotransmitter systems. For example, Glu receptors within the

NAc are able to mediate the release of acetylcholine (ACh) within the PFC (Zmarowski *et al*, 2007).

1.3.3.3 GABA

Like Glu, GABA is an amino acid neurotransmitter found throughout the CNS. However, in contrast to Glu, GABA is inhibitory in nature (except during gestation in humans and gestation and the first week of life in rats; see below). GABA is the primary inhibitory neurotransmitter in the mammalian CNS, playing an important role in the regulation of neuronal excitability throughout the CNS (Kumar and Kuppast, 2012). GABA receptors are widely distributed throughout the brain and have particularly high concentrations in the cortex, hippocampus, basal ganglia, thalamus, cerebellum, and brainstem (Kumar and Kuppast, 2012).

GABA receptors are classified into 3 subtypes; $GABA_A$, $GABA_B$ and $GABA_C$ (Tohyama and Takatsuji, 1998). $GABA_A$ and $GABA_C$ receptors are ionotropic receptors and exert their inhibitory effect by increasing the passage of Cl^- across the membrane (in the case of mature neurons). This results in the hyperpolarisation of the postsynaptic cell (Chebib and Johnston, 1999; Harris and Allan, 1985). The $GABA_A$ receptor directly gates the Cl^- ionophores; it is selectively blocked by the alkaloid bicuculline and has modulatory binding sites for benzodiazepines, barbiturates, neurosteroids and ethanol (Chebib and Johnston, 1999). Less is known about the $GABA_C$ receptor which is pharmacologically different than $GABA_A$ (Bormann and Feigenspan, 1995). There is currently some debate on the classification of $GABA_C$ receptors as to whether they should be considered their own class of GABA receptors or if they should simply be

considered a subclass of $GABA_A$ receptors (Bormann and Feigenspan, 1995; Bormann, 2000; Kumar and Kuppast, 2012).

$GABA_B$ receptors are metabotropic (coupled to G-proteins) and therefore indirectly alter membrane ion permeability and neuronal excitability. The activation of these receptor complexes produce a variety of effects including the inactivation of voltage dependent Ca^{2+} channels and the gating of K^+ ion channels. The $GABA_B$ receptors are widely distributed within the CNS and the peripheral nervous system and modulate the release of neurotransmitters by producing slow, prolonged inhibitory signals (Chebib and Johnston, 1999; Kumar and Kuppast, 2012).

There is a critical shift in the functioning of the GABA system that occurs during development. In the mature brain GABA is inhibitory, but in the developing brain GABA is often excitatory due to intracellular changes in Cl^- homeostasis. In immature neurons, the cotransporter for Cl^- (designated NKCC1) moves Cl^- into the cell, resulting in a high intracellular Cl^- concentration. As the neurons develops further, a different cotransporter (KCC2) is expressed which moves Cl^- out of the cell resulting in a lower intracellular Cl^- concentration. Because of this effect, the equilibrium potential for Cl^- is more positive in immature cells than it is in adult cells, and hence the opening of Cl^- channels is depolarizing such that GABA has the potential to excite the neurons to the point of firing (Ben-Ari *et al*, 2012; Cherubini *et al*, 1991). As brain development progresses, the equilibrium potential for Cl^- shifts such that GABA receptor activation results in a hyperpolarizing influx of Cl^-. It is believed that this shift changing GABA from an excitatory to an inhibitory transmitter is complete by the middle of the second week of postnatal life in the rat, although the precise age may be dependent on the sex of

the animal, with the shift being further delayed in males compared to females (Nuñez and McCarthy, 2007).

Considering the widespread role of GABA in the CNS, it is no surprise that GABA system dysfunction has been implicated in a variety of disorders including anxiety disorders, depression, autism and schizophrenia. Specifically, reductions in GABA transmission may be widespread in certain diseases like schizophrenia, potentially leading to a decrease in inhibitory neurotransmission (Rudolph and Möhler, 2014). Such changes in inhibition within the brain often result in alterations to attentional processing. A study by Pehrson *et al*, (2013) found that the blockade of $GABA_A$ receptors resulted in dose-dependent impaired performance in attentional processing. This finding is supported by other studies that also concluded that proper functioning of the GABA system, specifically with regard to the $GABA_A$ receptors within the PFC, is a required component in attentional processing (Murphy *et al*, 2012; Paine *et al*, 2011).

1.3.3.4 Other neurotransmitter systems

While a complete description of all the neurotransmitter systems implicated in attentional processing is beyond the scope of this thesis, it is important to recognise that other systems are involved. These include the norepinephrine, serotonin and cholinergic systems which are implicated to greater or lesser degrees, depending on the aspect of attentional processing in question (Evans and Drobes, 2009; Gasbarri and Pompili, 2014; Klinkenberg *et al*, 2011; Logue and Gould, 2013).

1.3.4 Methods for assessing attentional processing

As there are a variety of aspects to attentional and information processing, (outlined in section 1.3) so are there a variety of ways to test these phenomena. The test chosen depends on a number of factors including what is being assessed (e.g. sustained attention, selective attention, attention shifting) and the subject being tested (e.g. human, rodent) (Bushnell, 1998). As with any unobservable cognitive process, assessment of attention requires researchers to quantify something which can be observed and measured, such as behavior. The assessment of attention is further complicated because tests of attention typically measure more than one component of the process. Furthermore, attentional processing cannot be assessed in isolation without taking into account the sensory processes required to detect the incoming information and the response modality (e.g. motor or verbal) required to convey a result. Finally, many attention tasks overlap with other processes such as executive functioning and working memory. Because of these factors, many of the existing neuropsychological tests of attention actually assess a combination of attention, executive function, switching, inhibition and memory (Strauss *et al*, 2006).

A number of methods exist for assessing attentional and information processing in humans and other animals including eye tracking and orienting behavior, paper and computer based tests such as the Conners' Continuous Performance Test II (measures sustained attention and response inhibition) and the Paced Auditory Serial Addition Test (assesses working memory, divided attention and information processing speed). Behavioural tests are usually used in animals (see Bushnell and Strupp, 2009; Bushnell, 1998; Strauss *et al*, 2006 for review) although some like latent inhibition (LI) and PPI, (the two tests of attentional and information processing used in this thesis) can be

applied to both humans and other animals. Observed across many different species in including rats and humans, LI and PPI are reliably disrupted in a variety of neuropsychiatric disorders including schizophrenia (Baruch *et al*, 1988; Braff *et al*, 1978; Kohl *et al*, 2013) and have become widely used in studies of the neural alterations in schizophrenia as well as in the search for useful animal models of the disorder (Ellenbroek *et al*, 1996; Koch, 2013; Lubow, 1989; Moser *et al*, 2000; Zuckerman *et al*, 2003).

1.3.4.1 Latent inhibition

Latent inhibition is a normal cognitive process whereby previous non-reinforced experience with a particular stimulus impairs the ability of that stimulus to subsequently enter into new associations. According to Lubow (1989), who first proposed the conditioned attention theory of LI, when a conditioned stimulus (CS) is followed by no consequence, the animal learns to ignore that stimulus. As a result, during later pairing of the CS with an unconditioned stimulus (US) the animal fails to attend to the CS and associative learning is impaired. Latent inhibition is an important adaptive mechanism for the processing of incoming information as normally, the ability to ignore a stimulus that was irrelevant in the past would be beneficial (Lubow, 1997). Observed across many different species, including rats and humans (Lubow and Gewirtz, 1995), LI is reliably disrupted in humans with schizophrenia (Baruch *et al*, 1988) and has become widely used in studies of the neural alterations of various psychiatric disorders, as well as in the search for useful animal models of such disorders (Lubow, 1989, 2005; Moser *et al*, 2000). While early studies focused on experimental manipulations and disorders that produced a lack of LI, more recently it has been suggested that different aspects of

the observed changes in LI (namely the disruption of LI vs. the abnormal persistence of LI) in the clinical population and within animal models might illustrate different aspects and symptom categories of schizophrenia (Weiner and Arad, 2009; Weiner, 2003). The use of LI in rodent studies of psychiatric disease is discussed in greater detail in the Introduction to Chapter 3 of this thesis (section 3.1).

Latent inhibition can be assessed in a number of ways. In animals, the most common methods are through a conditioned taste aversion (CTA) task or a lick suppression conditioned emotional response (CER) task. In a CTA task the degree of aversion to an inert substance is measured both before and after that substance is paired with one that induces nausea. The animals will then display a different degree of interest in the inert substance, depending on the pre-exposure to that substance. In a lick suppression CER task, a particular stimulus such as a tone is paired with a mild shock that will affect the ability of the tone to suppress later drinking behaviour depending on the animal's previous, non-reinforced experience with the tone during the pre-exposure phase (Figure 1.2). In this case, the presence of LI is determined by a finding of significant differences in performance between the animals who received pre-exposure to the stimulus, and those that did not (Alves and Silva, 2001; Joseph *et al*, 1993; Lehmann *et al*, 2000; Lubow, 1989; Zuckerman and Weiner, 2003; Zuckerman *et al*, 2003).

Experimental data suggest that LI may be controlled by a neural circuit involving the hippocampus, the EC, the NAc, and the mesolimbic dopaminergic projection from the VTA to the NAc (Schmajuk *et al*, 2001; Weiner and Feldon, 1997; Weiner, 1990). While the precise neural circuitry underlying LI is not currently known, a small number

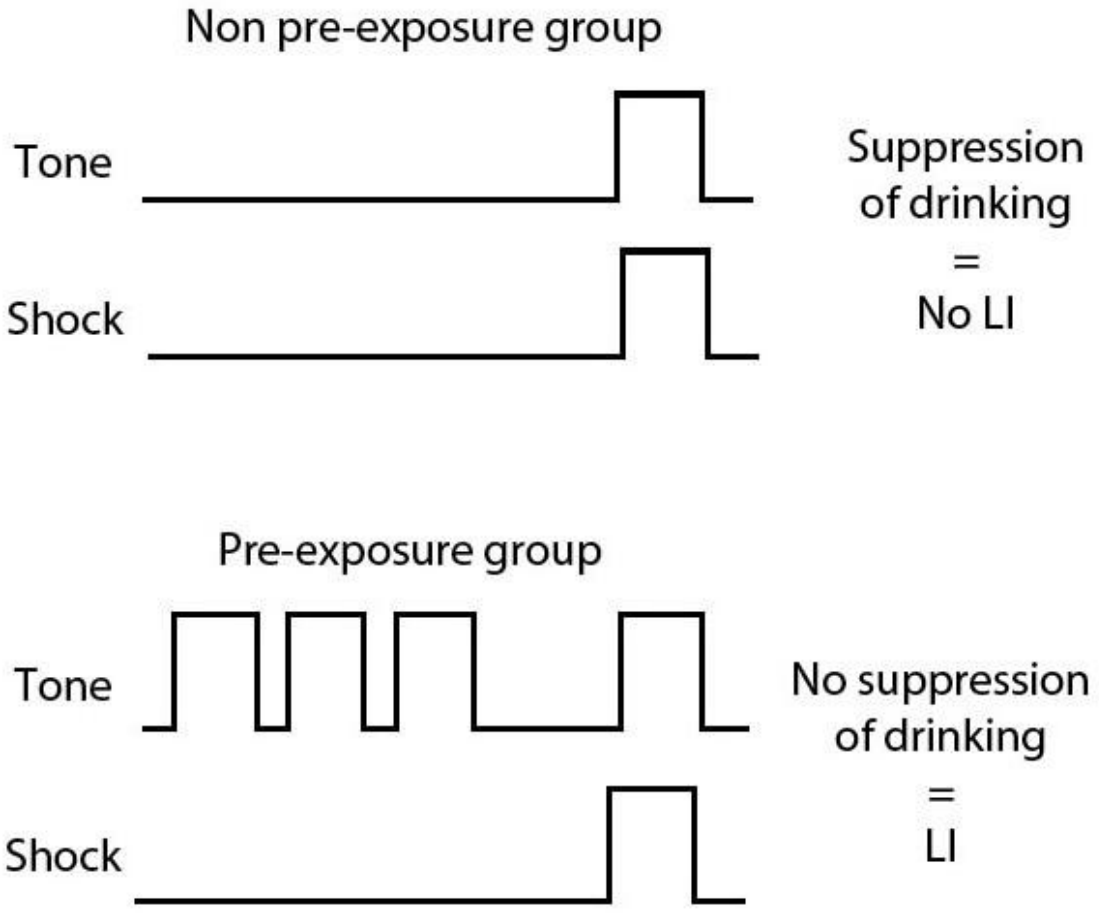

Figure 1.2 To assess LI using a lick suppression conditioned emotional response task, animals in the pre-exposure (PE) group receive experience with the tone stimulus, while the animals in the non pre-exposure (NPE) group do not. All animals received pairings of the tone with a foot shock. The normal resulting behavior is that, when tested later on, animals in the NPE group will display suppressed drinking behaviour when a tone is played (no LI). The animals in the PE group will display no/reduced drinking suppression when a tone is played (LI).

of theoretical models have been proposed with the most well characterised being the Schmajuk-Lam-Gray (SLG) model proposed by Schmajuk *et al*, (1996) and the switching model proposed by Weiner, (1990).

A simplified diagram of the SLG model is illustrated in Figure 1.3. According to this model, a variety of implicated brain areas are connected by excitatory Glu projections as well as inhibitory GABA and DA projections. The SLG model thus focuses on using a variety of techniques including lesion studies, pharmacological manipulations and computer modeling to determine the neurochemical associations between the various brain regions and how such connections may influence LI (Schmajuk *et al*, 1996, 2001).

According to this model, LI is produced due to a series of events beginning when the animal first experiences the non-reinforced stimulus. Incoming sensory information leads to increased activity within the EC and thus the hippocampus, as the prediction of the CS by context increases. This leads to increased activity within the shell of the NAc, which leads to decreased activity within the VTA. Decreased activity within the VTA leads to a decrease in the thalamus via two pathways, firstly due to a decrease in activity in the core of the NAc, and thus, an increase in activity within the ventral pallidum, which leads to a decrease in the activity in the thalamus. Secondly, DA connections from the VTA to the thalamus also contribute to decreased activity. This decreased activity in the thalamus produces a retarding of the formation of the CS-US association within the amygdala (Schmajuk *et al*, 1996, 2001). The experiments used to construct this model utilized conditioned suppression experiments (similar to the ones used in Chapters 2 and 3 of this thesis), thus LI was evaluated based on the amount of behavioural suppression observed in the presence of a tone CS. Since this suppression

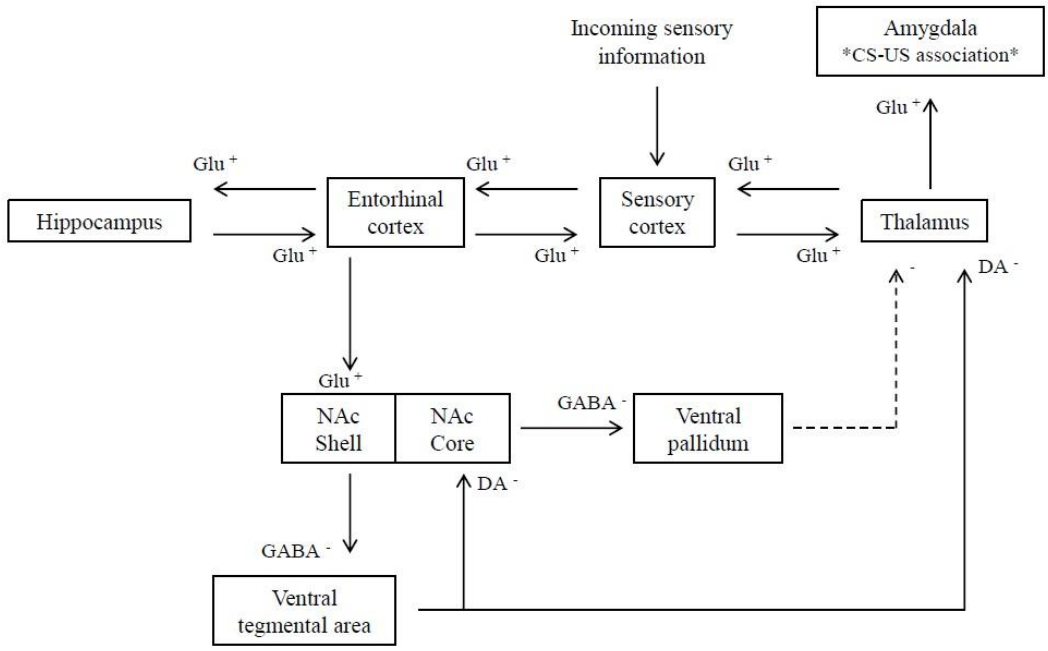

Figure 1.3 A simplified diagram illustrating the neural circuitry of the SLG model of attentional processing in latent inhibition. Brain areas are represented by boxes and the putative connections between those areas are illustrated by arrows with implicated neurotransmitter system stated. Solid lines indicate direct connections, dashed lines indicate indirect actions. Neurotransmitters include glutamate (Glu), dopamine (DA) and GABA. Excitatory pathways are designated by a +, inhibitory pathways are designated by a -. The nucleus accumbens is designated by NAc. Adapted from (Schmajuk *et al*, 2001).

theoretically reflects the strength of fear response, it was postulated that the strength of the conditioned fear response was regulated within the amygdala (Killcross *et al*, 1997).

The switching model, first proposed by Weiner (1990) is another theoretical model of LI circuitry. However, in contrast to the SLG model which focuses primarily on neural connections, the basis of the switching model is that LI is controlled by a "switching mechanism" executed within the NAc, based on inputs from a variety of brain areas (see Figure 1.4). The model was proposed based on earlier findings which indicated that administration of drugs that affect the DA system also showed an effect on LI, but only when administered during the conditioning phase and not the pre-exposure phase (Weiner *et al*, 1981, 1984, 1988). It was thus proposed that an explanation of LI behaviour cannot be limited to processes that occur during the initial exposure to a CS, but may be due to separate and/or additional processes occurring during the conditioning phase of the CS-US pairing (Weiner, 1990).

According to this model, when an animal experiences a situation with the potential to produce LI, they experience two independent and conflicting experiences; stimulus-no event (during pre-exposure) and stimulus-reinforcement (during conditioning). These experiences compete for expression. So while the animal may learn that the stimulus is irrelevant during pre-exposure, it is during the conditioning stage that the irrelevance is manifested and the animal (if demonstrating LI) continues to treat the stimulus as irrelevant despite the fact that it now signals an outcome (Weiner, 2003). Latent inhibition is therefore the result of the animal remaining (during conditioning) under the control of the "CS- no US" relationship established during the pre-exposure phase.

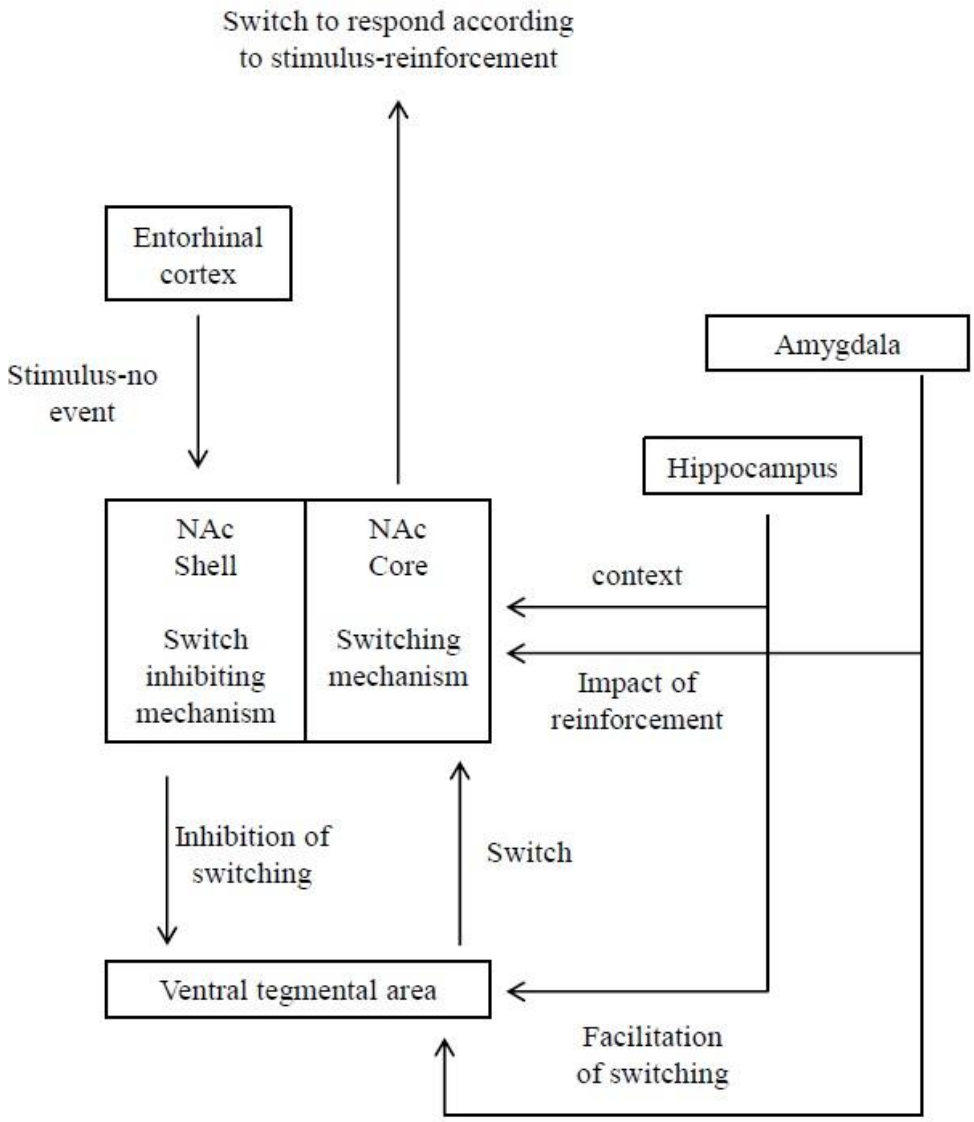

Figure 1.4 A simplified diagram illustrating the brain regions and pathways involved in LI according to the switching model. Adapted from (Weiner, 2003).

The hippocampus and the NAc are the primary brain areas implicated in the switching theory, with the hippocampus computing the association of the incoming stimulus and then either inhibiting or activating a mechanism within the NAc which "switches" the animal's behaviour, based on the context determined by previous behaviour with that stimulus. If the animal has previous experience with a stimulus, the hippocampus assigns a low attentional processing value to the stimulus, inhibiting the switching signal of the NAc. In this case, the animal will continue to respond to the stimulus according to past experience, namely, that there is no CS-US association. However, if the input from the hippocampus indicates a different context (lowering the effect of stimulus pre-exposure), or if the input from the amygdala indicates a strong relationship between the CS and US (higher association) then the switching signal within the NAc will be activated and the animal will respond according to stimulus-reinforcement (Weiner and Feldon, 1997; Weiner, 1990, 2003).

While it shares many similar characteristics with the SLG model (namely many of the brain areas and primary neurotransmitter systems believed to be involved) the switching model is unique in that it offers a potential explanation for the two types of LI abnormalities that can be observed in experimental animals (the disruption of LI and the abnormal persistence of LI). Inherent in the explanation provided by the switching model for this phenomenon is the theory of functional differences between the core and the shell of the NAc and their role in LI behaviour. Namely, that the pathway from the EC to the NAc shell is responsible for the "no switch" signal, which is responsible for inhibiting the switching mechanism of the NAc core, through the VTA (see Figure 1.4). Support for this theory is indicated by the finding that damage to the NAc shell disrupts LI and damage to the core produces abnormally persistent LI. Furthermore, this

occurrence is imitated by damage to the areas of input, where damage to the EC disrupts LI and damage to the hippocampus and the amygdala produce abnormally persistent LI (Weiner, 2003). Finally, the importance of DA functioning within the NAc is pivotal to the switching theory of LI circuitry, as the disruption of LI caused by the administration of amphetamine is due to the increased activation of the NAc, and the resulting activation of the switching response (thus identifying a relationship between the CS and US). Consequently, DA blockade results in the abnormal persistence of LI by preventing the elimination of the LI behaviour by blocking the "switching" ability (Weiner, 2003).

1.3.4.2 Prepulse inhibition

Prepulse inhibition is the normal suppression of the startle reflex that occurs when the startling stimulus (usually auditory) is preceded by a less intense, non-startling stimulus (see Figure 1.5) (Graham, 1975). This behaviour can be used to detect deficits in sensorimotor gating suggestive of the inability to properly filter incoming sensory information, which is an early stage of attentional processing (sometimes called pre-attentional processing, see section 1.3) (Hazane *et al*, 2009; Swerdlow *et al*, 2000; Uehara *et al*, 2009). It has been proposed that PPI is due to a gating mechanism that protects the ongoing processing of the present stimulus from disruption by other incoming stimuli (Koch and Schnitzler, 1997). Prepulse inhibition changes have been observed in a number of clinical disorders including Huntington's disease (Swerdlow *et al*, 1995), seizure disorders (Pouretemad *et al*, 1998), Tourette syndrome (Castellanos *et al*, 1996; Swerdlow *et al*, 2001b), OCD (Swerdlow *et al*, 1993) and schizophrenia (Braff *et al*, 1978; Ludewig *et al*, 2003; McDowd *et al*, 1993; Parwani *et al*, 2000).

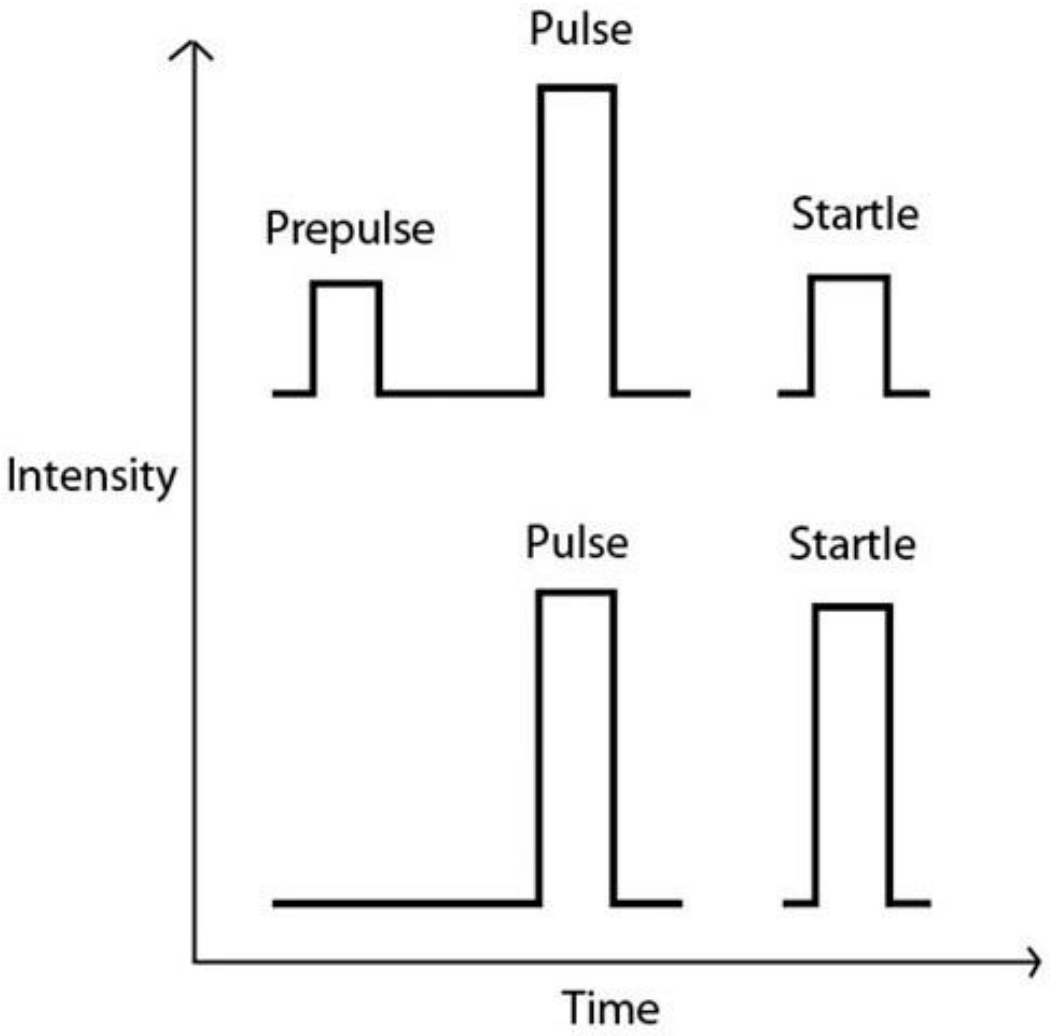

Figure 1.5 Prepulse inhibition in a normal animal. Startle is lowered when the pulse is preceded by a prepulse.

The behavioural response to auditory startle itself is controlled by structures located in the brainstem, but the inhibition of the startle circuit is believed to be mediated by input from the forebrain (Weiss and Feldon, 2001). While the precise brain mechanisms underlying the mediation of PPI are not known, a number of models have been proposed (Fendt *et al*, 2001; Koch and Schnitzler, 1997; Koch, 1999; Li *et al*, 2009; Swerdlow *et al*, 2000, 2001a). Although by no means a complete description of the brain areas and systems involved in this complex behaviour, Figure 1.6 illustrates the theoretical circuitry of the acoustic startle response and its inhibition by an auditory prepulse. According to this model, the relatively simple startle circuit (consisting of the cochlear nuclei, the caudal pontine reticular nucleus and the subsequent interneurons and motor neurons) receives input from the pedunculopontine tegmental nucleus in the presence of a non-startling prepulse, which receives inhibitory input from a variety of forebrain structures, primarily via the NAc. While the current knowledge of the specific role of some of these forebrain structure (such as the amygdala) on PPI is more limited, other regions such as the mPFC, the hippocampus and the NAc have been more thoroughly investigated.

The involvement of the mPFC in the regulation of PPI is consistent with the proposed role of reduced PFC DA activation in schizophrenia. One explanation for this disruption is that decreased dopaminergic activity in the mPFC is linked to increased dopaminergic activity in the NAc via disinhibition of descending glutamatergic fibers (Koch and Bubser, 1994; Swerdlow *et al*, 2001a). This is consistent with the theory that many of the symptoms of schizophrenia are linked to increased DA activity in the mesocorticolimbic system, decreased DA activity in the PFC, and that these DA

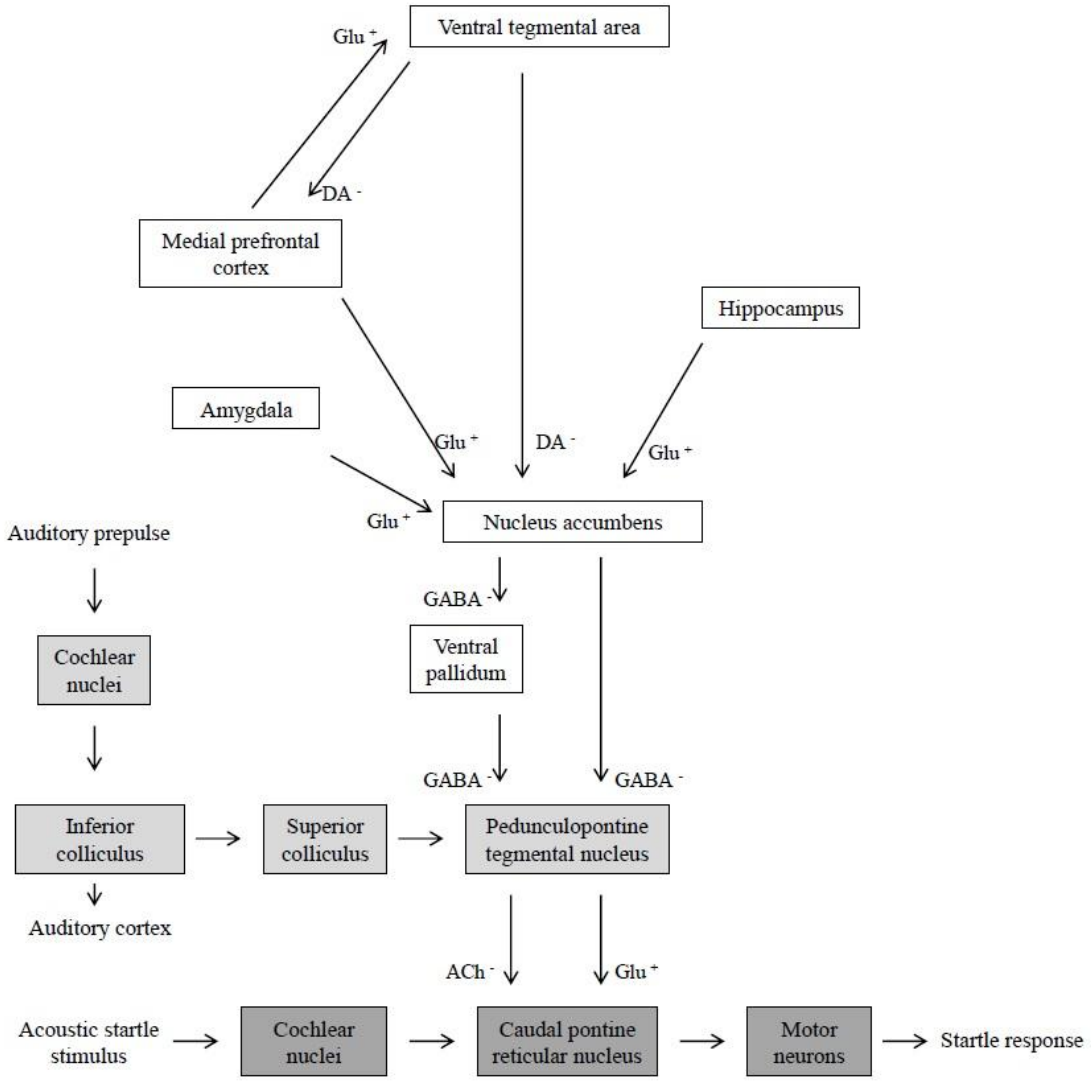

Figure 1.6 A hypothetical model of the neural circuitry regulating the acoustic startle response and PPI. Shaded boxes indicate the brainstem structures responsible for the primary startle circuit (darker boxes) and the processing of normal auditory information/ mediation of prepulse information (lighter boxes). White boxes indicate the forebrain structures responsible for the modulation of PPI though inhibitory input. Arrows illustrate the connections between areas as well as the neurotransmitters and action (inhibitory or excitatory), if known. Based on models proposed by (Fendt *et al*, 2001; Koch and Schnitzler, 1997; Koch, 1999; Li *et al*, 2009; Swerdlow *et al*, 2000, 2001a).

abnormalities are regulated, at least in part, by abnormal glutamate function (see sections 1.2.5.1 and 1.2.5.2).

Studies with animals have suggested that the hippocampus is capable of regulating PPI through its glutamatergic connections with other areas of the brain. A pathway of particular importance to the PPI circuitry is believed to be the Glu projections from the ventral hippocampus to the NAc (Bast and Feldon, 2003). The NAc is a major hub in the transfer and translation of information flowing between the limbic and cortical structures and has also been strongly implicated in PPI. The NAc has glutamatergic connections with the hippocampus, mPFC, and amygdala, as well as DA connections with the VTA. While the exact nature of the interactions are not yet understood, it is believed that interaction between Glu and DA within the NAc are important for modulating PPI. Increased DA in the NAc is typically found in animal models of schizophrenia, and the level of DA in the NAc is increased when the subject is exposed to DA agonist drugs such as apomorphine, which also alter PPI (reviewed by Koch and Schnitzler, 1997; Swerdlow *et al*, 2001a).

While deficits in PPI are not likely to cause concern themselves, they are indicative of potential dysfunction in a number of brain areas and systems (Swerdlow and Geyer, 1998). Furthermore, the inability to properly process incoming sensory information could lead to the emergence of other schizophrenic behaviours, such as those seen in the positive category of symptoms (Braff and Geyer, 1990).

1.4 Domoic acid

Domoic acid is a naturally occurring environmental toxin produced by various marine organisms such as the red algae *Chondria armata* and a variety of phytoplankton

of the Nitzshia *spp*. (for review see Bates, 1988; Doucette and Tasker, 2008). The toxin enters the food chain by accumulating in filter feeding animals that are subsequently consumed by other organisms, including humans. While various shellfish and crustaceans can become contaminated with DOM, it is most commonly found in the blue mussel (Pulido, 2008).

In Canada during 1987 a major human poisoning incident occurred when over 100 people became seriously ill after consuming mussels contaminated with DOM (Perl *et al*, 1990). The syndrome was termed "amnesic shellfish poisoning" and was characterized by symptoms which included vomiting, abdominal cramps, diarrhea, headache, confusion, disorientation and loss of short term memory. In some patients, the memory deficits were found to be persistent and were still present 4-6 months after DOM exposure (Teitelbaum *et al*, 1990). Those patients who were affected most severely experienced seizures, coma, respiratory distress, unstable blood pressure and even death (Perl *et al*, 1990). Post mortem studies on four people who died as a result of DOM exposure found damage to the hippocampus in the form of pyramidal neuron cell loss (Teitelbaum *et al*, 1990).

Structurally similar to Glu, DOM can induce excitotoxicity by acting on both pre and post-synaptic Glu receptors. At low concentrations DOM is selective for KA receptors, in particular the low-affinity KA receptors, although at higher concentrations other receptors are also able to be activated (Tasker *et al*, 1996; Verdoorn *et al*, 1994).

Most of the work with DOM has focussed on toxicity and neurotoxicity (for review see Costa *et al*, 2010; Jeffery *et al*, 2004). Of particular relevance to this thesis, a study by Sobotka *et al*, (1996) investigated the neurotoxic potential of DOM by administering 0.22, 0.65 and 1.32 mg/kg of DOM to adult male and female rats through

intraperitoneal (i.p.) injection. Rats who received the highest dose (1.32mg/kg) displayed symptoms of toxicity with approximately 25% dying or being euthanized. Although minimal behavioural changes were seen in the surviving animals when behavioural testing began (1 day after dosing), animals who received the higher dose of DOM displayed a significantly exaggerated startle response. An examination of the brains of these animals revealed that a small number of animals displayed degeneration of hippocampal neurons in the CA1 and CA3 regions.

1.4.1 Domoic acid exposure during development

While the neurotoxic effects of DOM administration on the adult CNS have been well studied, the effects of DOM on the developing CNS are also of interest, particularly because the potentially toxic effects of EAAs and their agonists differ between the mature and the still developing brain. Indeed, a growing body of evidence suggests that the developing CNS may be more susceptible to the toxic effects of DOM. A study by Doucette *et al*, (2000), demonstrated that the potency of DOM is much higher at younger ages, with 5 day old rats requiring half the dose of DOM to achieve the same level of behavioural toxicity as 14 day old animals (1/17 of the adult dose).

1.4.1.1 Prenatal exposure

A small number of studies have looked at the effects of prenatal DOM exposure. Dakshinamurti *et al*. (1993) showed that intrauterine exposure to DOM caused hippocampal alterations that progress with age. By injecting 0.6 mg/kg of DOM into the caudal vein of pregnant mice on gestational day 13, they found that litter size, birth weight and hippocampal structure did not differ from control on postnatal day (PND) 1

but that morphological changes in the hippocampus were observed beginning on PND 14. Neurochemical changes were also seen with an increase in KA receptors, Glu levels, and Ca^{2+} influx, as well as a decrease of GABA found in the hippocampus. Mice also displayed a reduced seizure threshold to a subsequent dose of DOM (Dakshinamurti *et al*, 1993). Levin *et al*. (2005), reported that a single prenatal exposure to DOM during midgestational development (with doses ranging from 0.3-1.2 mg/kg) produced dose related changes which included an increase in response latency in a spontaneous alternation task in the T-maze, as well as an increase in initial activity and a larger habituation score in the Figure-8 maze. In a more recent study by Tanemura *et al*. (2009), mice were given a single injection of DOM (1.0 mg/kg) on gestational day 11.5, 14.5 or 17.5 and pups underwent a series of behavioural tests between 4 and 11 weeks of age. A variety of behavioural changes were observed including impairments in learning and memory and an increase in anxiety like behaviours, with results depending on the gestational day of DOM administration.

1.4.1.2 Postnatal exposure

Studies have also investigated the effects of DOM during the postnatal period. A number of studies using doses of DOM ranging from 0.05-1.5 mg/kg have been found to produce increasingly severe seizure behaviour including scratching, convulsions, electroencephalograph (EEG) alterations and death in neonatal rats (Wang *et al*, 2000; Xi *et al*, 1997). Another study by Levin *et al*. (2006), considered the effect of a range of DOM doses (0.025-0.1 mg/kg) on rat pups when administered twice per day, on PND 1 and 2. While the higher doses proved to be lethal, they did report some effects at lower

doses, but these were less frequently observed than those seen following DOM exposure during mid-gestation (see section 1.4.1.1).

A large series of studies in the Tasker, Ryan and Doucette labs at UPEI have investigated the behavioural, histological and histochemical effects of repeated low dose DOM exposure during a critical period of CNS development. Our protocol consists of a single daily subcutaneous (s.c.) injection of 20 µg/kg of DOM administered to Sprague-Dawley rat pups from PNDs 8-14. This is a rapid period of brain growth and change which is considered to be a critical period of development for the CNS (Dobbing and Smart, 1974). Using this protocol, Doucette *et al*. (2003) demonstrated that this low dose of DOM did not produce overt behavioural toxicity. Domoic acid treated rats were later reported to show increased conditioned place preference in an olfactory conditioning paradigm, indicating that this low dose of DOM was biologically relevant and centrally active.

Further studies at UPEI using the same low dose DOM treatment have shown that this protocol results in a wide variety of behavioural and histopathological changes that progress as the animals age. Many of these changes have relevance to understanding the process of epileptogenesis, such as seizure-like behaviour in response to a novel spatial environment (Doucette *et al*, 2004), reductions in both general and focal seizure threshold (Gill *et al*, 2010a) and seizure-related changes in hippocampal morphology such as mossy fibre sprouting (Bernard *et al*, 2007; Doucette *et al*, 2004). Of particular relevance to this thesis are a number of findings indicating the potential usefulness of this neonatal DOM treatment to model certain aspects of neuropsychiatric illness. Specific alterations found in this model to brain measures known to also be affected in schizophrenia include increases in hippocampal BDNF messenger ribonucleic acid

(mRNA), increases in hippocampal mossy fiber sprouting, a decrease in hippocampal cell counts and elevated trkB receptor expression (Bernard *et al*, 2007; Doucette *et al*, 2004). The studies illustrating the behavioural findings relevant to the modeling of neuropsychiatric disease are reviewed in detail in the Introductions to Chapter 3 (section 3.1) and Chapter 4 (section 4.1).

1.5 Animal models of schizophrenia and related disorders

Current research on neuropsychiatric diseases like schizophrenia is conducted by a variety of methods including genetic analyses, imaging studies, work within the clinical population, as well as the post-mortem analyses of patients. While much has been gained though these methods, the research of such disorders through the investigation of animal models also has much to offer. Currently, there are a number of animal models of schizophrenia that have aided researchers in the understanding and treatment of this disease as well as related diseases. However, many questions still remain, particularly with regard to neuropsychiatric disorders where the precise etiology and/or resulting brain dysfunction is not completely understood. Because of this lack of knowledge, our ability to successfully treat these disorders is compromised. Additionally, the more we learn about a disorder, the more our understanding of the disorder changes. It is not surprising, therefore, that what we require from animal models is also constantly changing and accordingly, so is their usefulness and applicability. New animal models are thus required in order for research to continue to advance and to further our understanding of these debilitating illnesses so that we may better aid those afflicted.

Animal models can be thought of as preparations developed for the purpose of studying a condition in the same or potentially in a different species. A variety of animals are used for modeling human disorders with the most common being rats and mice. Such models can be used to mimic a human condition in a variety of different ways, depending on the nature and purpose of the model (Geyer and Markou, 2000). Animal models provide a valuable way to study many complex human disorders. In addition to more obvious clinical applications such as testing new treatments or medications, they can also be used to study the underlying neurobiological mechanisms that contribute to the human disorder.

Neuropsychiatric disorders often provide a unique challenge to model in animals since diagnostic criteria requires symptoms than cannot generally be directly assessed in animals (see section 1.2.1 for the diagnostic criteria of schizophrenia). However, while it may not be possible to create a complete animal model of a complex human disorder (due to the variability in symptom manifestation as well as the human aspect of some symptoms, such as auditory hallucinations), many of the core symptoms of schizophrenia have been successfully produced in animals. Improvements on current models as well as the development of new models have the potential to add significantly to our understanding of many aspects of schizophrenia (e.g. the role of genetics, molecular basis, therapeutic strategies) and will undoubtedly benefit human clinical practice (VanDenBuuse *et al*, 2005). Current animal models of schizophrenia can generally be grouped by type into genetic, surgical, pharmacological and developmental/environmental categories. It is, however, important to note that these categories are not necessarily mutually exclusive and that some models, although

generally placed with one particular group, may actually have characteristics of multiple groups.

1.5.1 Genetic models

A large number of genes have been identified as being "candidate genes" for schizophrenia. As a result, the number of animal models of schizophrenia which are genetic in nature is staggering (see Carpenter and Koenig, 2008 for review). Genetic mouse models are designed to investigate these genes of susceptibility with targeted mutations, which allow researchers to identify the functional significance of the gene and test the behavioural phenotype that emerges (O'Tuathaigh *et al*, 2007).

One example of a candidate gene currently under investigation is DISC1. An assortment of genetic linkage and association studies support DISC1 as a susceptibility gene for a variety of neuropsychiatric disorders with depression and schizophrenia being at the forefront (Cash-Padgett and Jaaro-Peled, 2013). First proposed by (Millar *et al*, 2000), mice used for this model have displayed enhanced locomotor activity (Hikida *et al*, 2007; Pletnikov *et al*, 2008), disrupted PPI (Hikida *et al*, 2007) and impaired social interaction (Pletnikov *et al*, 2008). Enlarged ventricles were also found in the brains of these animals, particularly on the left side (Hikida *et al*, 2007; Pletnikov *et al*, 2008). Some of the other more popular models making use of potential candidate genes include the NRG1 hypomorph (Stefansson *et al*, 2002), the DTNBP1 mouse (Straub *et al*, 2002) and the COMT knockout (Egan *et al*, 2001).

Although genetically based animal models of schizophrenia are undoubtedly helpful, one must keep in mind that it is believed that in the majority of cases, genetic factors alone are not sufficient to produce the disease and therefore can only be a part of

the cause. Additionally, many proposed candidate genes offer only a loose connection with the disorder, highlighting the importance of non-genetically based models.

1.5.2 Surgical models

The most commonly studied and widely accepted surgical model of schizophrenia is the neonatal ventral hippocampal (NVH) lesion model. First proposed by Lipska *et al.* (1993) the NVH lesion model uses ibotenic acid (a Glu agonist) to lesion the ventral hippocampus of neonatal rats on PND 7 of development. Rats receiving the lesions display many aspects of the disorder when compared to control animals. Lesioned rats demonstrated a higher degree of activity in response to a novel environment and an amphetamine challenge in a novel environment, when tested in early adulthood (PND 56) but not at an earlier time point (PND 35) (Lipska *et al*, 1993). Deficits have also been observed in other behavioural measures including PPI (Lipska *et al*, 1995), LI (Grecksch *et al*, 1999), measures of working memory (Lipska *et al*, 2002) and social interaction, with rats who received NVH lesions spending less time in interacting socially compared to controls (Becker *et al*, 1999). Neurobiological changes such as an increase in hippocampal DA have also been found (Alquicer *et al*, 2004). In addition, antipsychotic drugs normalize some of the lesion-induced behaviors including the blockade of increased motor activity in a novel environment by haloperidol (Lipska *et al*, 1993) and an increase in the amount of time spent in social interaction following treatment with clozapine, but not with haloperidol (Becker and Grecksch, 2003).

Other surgically-based models include the Neonatal Amygdalar Lesion Model (Daenen *et al*, 2003) where ibotenic acid is used to lesion the amygdala on PND 7, and

the Prefrontal Cortical Lesion Model (Miner *et al*, 1997) which uses ibotenic acid to lesion the PFC in neonatal or adult animals.

Both surgically-based models and chemically-based models have successfully illustrated many aspects of schizophrenia, thereby fulfilling many issues of validity. The various models have succeeded in producing behavioural, neurochemical and neuroanatomical changes similar to those seen in the clinical population, and a number of these changes have been shown to be reversible with antipsychotic medications. However, the issue of etiological validity remains, as many of the models do not mimic the etiology of the clinical condition.

1.5.3 Pharmacological models

1.5.3.1 Amphetamine

One of the earliest animal models of schizophrenia was that of amphetamine administration in rats. This model, proposed by Gambill and Kornetsky (1976) involved adult animals being given daily i.p. doses of increasing amounts of amphetamine. A more recent model, proposed by Abekawa *et al*, (2008) used repeated 2.5 mg/kg doses of methamphetamine to produce symptoms of schizophrenia in adult rats. This paradigm resulted in increased Glu levels in the medial PFC as well as disruptions to PPI. These PPI deficits were able to be reversed following treatment with olanzapine and resperidone (Abekawa *et al*, 2008). Other similar procedures have resulted in an upregulation of mGluR8 mRNA in the caudate putamen and NAc (Parelkar and Wang, 2008), a decrease in glutamic acid decarboxylase (GAD)67 in the hippocampus, PFC, thalamus and amygdala, as well as behavioural alterations including changes to PPI and LI (Peleg-Raibstein *et al*, 2008).

1.5.3.2 NMDA receptor antagonism

Pharmacological blockade of NMDA receptors in adult animals has gained popularity as a model of schizophrenia following the implication of Glu in the disorder (see section 1.2.5.2). Both acute and chronic treatment with non-competitive NMDA antagonists like phencyclidine (PCP) and MK-801, will reliably produce a variety of schizophrenia-like symptoms in both animals and humans (Morris *et al*, 2005; Pouzet *et al*, 2005; Rung *et al*, 2005; Turgeon and Hoge, 2003). Administration of such compounds has also been found to exacerbate schizophrenia symptoms within the clinical population (Javitt and Zukin, 1991; Morris *et al*, 2005).

In animals, the PCP model results in a variety of behavioural changes indicative of schizophrenia including hyperlocomotion (Adams and Moghaddam, 1998), deficits in PPI (Geyer *et al*, 1984), social withdrawal (Sams-Dodd, 1997), and changes to spatial learning and memory (Wass *et al*, 2006). Elevated levels of both DA and Glu in the PFC and the NAc after PCP administration have also been found (Adams and Moghaddam, 1998). It is important to point out however that some of these symptoms have a very specific window of detection, making the observation of the behavioural and neurochemical changes outlined above dependent on the time that has elapsed following drug administration (Adams and Moghaddam, 1998).

Phencyclidine-induced behaviour changes have been shown to respond to some, but not other, currently prescribed antipsychotic drugs. While clozapine has been shown to successfully block PCP-induced hyperlocomotion (Freed *et al*, 1980), restore social behaviour (Steinpreis *et al*, 1994) and restore PPI deficits (Bakshi *et al*, 1994; Swerdlow *et al*, 1996), these same effects were not observed following haloperidol treatment

(Freed *et al*, 1980; Harte *et al*, 2007; Steinpreis *et al*, 1994) which did not produce any effect or did not ameliorate the behaviours to the same extent.

1.5.4 Developmental/ environmental models

Although schizophrenia generally arises in adolescence or early adulthood, it is increasingly regarded as being neurodevelopmental in origin (see section 1.2.3). It is believed that subtle perturbations in the developing brain result in a permanent change in brain development, increasing the risk of developing schizophrenia later in life.

Developmental models of disease rely on the concept of critical periods in brain development. In brief, the nervous system is more vulnerable to insult during development than it is during adulthood. The reason for this is that during development the nervous system is undergoing a multitude of sensitive processes including neuronal proliferation, migration, differentiation, synaptogenesis, mylenation and apoptosis. During times of such rapid growth and change, even very small alterations to the normal process of events can have a significant and long lasting effect on the developing organism, potentially altering the functioning of the CNS for life (Rice and Barone, 2000). These critical periods of developmental sensitivity are not limited to the prenatal period but extend into adolescence (Dobbing and Smart, 1974; Kaufmann, 2000; McDonald and Johnston, 1990; Vorhees, 1986). While in some cases no outward changes in behaviour or development are observed, current theories indicate that many disorders may be due, at least in part, to subtle changes during critical periods of development.

Two of these potential perturbations, exposure to infection and environmental stress, are the most commonly used in developmental animal models of the disorder

(Cash-Padgett and Jaaro-Peled, 2013). More recently, a new neurodevelopmental model has been introduced; the neonatal domoate model was developed at UPEI and was used in the studies described in this thesis.

1.5.4.1 The polyI:C model

Prenatal exposure to infection (both of the mother and of the fetus) has been implicated in an increased risk of developing mental disorders. Presently it is believed that the maternal immune response to infection, particularly the pro-inflammatory cytokines that are released in such situations, can interfere with the normal development of the CNS and lead to increased risk of schizophrenia later in life (Brown *et al*, 2004a).

Immunologically-based models seek to imitate this potential risk factor (Zuckerman and Weiner, 2005). Seeking to elicit the maternal immune response by mimicking viral exposure, Zuckerman and Weiner (2003) created the polyI:C model whereby pregnant rats are injected with polyriboinosinic-polyribocytidilic acid. The resulting adult animals display a variety of schizophrenia-like changes in behaviour including PPI deficits (Ozawa *et al*, 2006), LI deficits (Zuckerman and Weiner, 2003), increased sensitivity to the locomotor effects of amphetamine (Zuckerman *et al*, 2003), impairments in novel object recognition (Ozawa *et al*, 2006) and reduced social interaction (Shi *et al*, 2003). Neuroanatomical and neurochemical abnormalities have also been observed in this model, including alterations to hippocampal morphology (Zuckerman and Weiner, 2003), and increases in striatal DA release (Zuckerman *et al*, 2003). Additionally, it has been found that both haloperidol and clozapine are able to reverse the LI effect (Zuckerman *et al*, 2003) but only clozapine improved novel object recognition (Ozawa *et al*, 2006).

1.5.4.2 The neonatal quinpirole model

Repeated administration of the D2/D3 receptor agonist quinpirole early in development results in a long term increase in D2 receptor sensitivity. As a result of this treatment, adult animals display a variety of behavioural and neurological changes in adulthood consistent with schizophrenia and potentially, with other disorders that implicate DA dysfunction (Brown *et al*, 2004b, 2004c; Kostrzewa and Brus, 1991; Kostrzewa, 1995; Nowak *et al*, 2001).

In a series of studies using this model, it was found that when given repeated doses of quinpirole during the neonatal period (1mg/kg/day i.p. from PND 1-21), male and female Sprague-Dawley rats displayed increased behavioural sensitization illustrated by hyperlocomotion, increased yawning, and increased jumping (Kostrzewa and Brus, 1991; Kostrzewa, 1995; Kostrzewa *et al*, 1990, 1993a, 1993b). Other behavioural effects include deficits in the MWM and in a skilled reaching task (Brown *et al*, 2002, 2004b, 2004c, 2005; Thacker *et al*, 2006). This neonatal quinpirole treatment protocol produces a significant decrease in nerve growth factor (NGF) in the hippocampus and PFC, as well as decreases in BDNF and acetyltransferase in the hippocampus and PFC (Brown *et al*, 2006; Thacker *et al*, 2006). Furthermore, some of the reported behavioural and neurochemical alteration have been found to be partially or totally blocked by the administration of the antipsychotic olanzapine (Brown *et al*, 2008; Thacker *et al*, 2006) as well as by the administration of nicotine (Brown *et al*, 2004b, 2004c, 2006; Tizabi *et al*, 1999).

1.5.4.3 Environmental stress models

A stressful environment can cause maladaptive brain development and functioning from the period of prenatal development up until adulthood. These effects can be modeled in animals in a variety of ways. Some stress paradigms such as mild chronic stress (Hill *et al*, 2012) and social defeat (Kudryavtseva *et al*, 1991; Venzala *et al*, 2012) are used most often to model depression in animals, while neonatal maternal separation (Schmidt *et al*, 2011) and social isolation rearing are used to model schizophrenia (see section 1.5.4.5 for more on isolation rearing).

1.5.4.4 Neonatal domoate model

The neonatal domoate rat model uses repeated systemic injections of low doses of DOM, a Glu receptor agonist, to stimulate the Glu system of rats during the second postnatal week of life. Although the treatment produces no overt symptoms in rat pups, once the animals reach adulthood they display a host of behavioural, neuropathological and neurochemical alterations, many of which are progressive in nature as the animals age. While initially investigated as a potential animal model of temporal lobe epilepsy (Bernard *et al*, 2007; Doucette *et al*, 2004, 2007; Gill *et al*, 2009, 2010a, 2010b; Perry *et al*, 2009), more recent studies have found that the same protocol of neonatal DOM treatment can be used to model many of the characteristics of schizophrenia (Adams *et al*, 2008; Marriott *et al*, 2012). This animal model was discussed in section 1.4.1.2 and used throughout the work described in this thesis.

1.5.4.5 Social isolation rearing

Since Hatch *et al*, (1963) first reported that housing rats in isolation produced abnormal behavioural reactivity, many studies have shown that rats who experience social isolation (housed one animal per cage for some period of time post-weaning, still in auditory, visual and olfactory contact with other animals) display a variety of profound behavioural, neurobiological and neuroanatomical differences when compared to those rats who are raised in groups (Ferdman *et al*, 2007; Hall, 1998; Lehmann and Feldon, 2000; Paulus *et al*, 1998; Weiss *et al*, 2004).

Examples of behavioural alterations include locomotor hyperactivity (Einon and Morgan, 1978; Gentsch *et al*, 1988; Heidbreder *et al*, 2000), spatial working memory impairments (Einon, 1980) increased food hoarding behaviour (Heidbreder *et al*, 2000), impairments in reversal learning (Jones *et al*, 1991), a modified response to reward (Wongwitdecha and Marsden, 1995) and increased sensitivity to amphetamine (Jones *et al*, 1990). However, results indicate that these effects may vary according to the timing of the isolation, the timing of the testing period, the strain of rat used, and the other experiences of the animal (Gentsch *et al*, 1988; Han *et al*, 2012; Shao *et al*, 2009; Weiss *et al*, 2001; Wilkinson *et al*, 1994).

A number of studies have also demonstrated that social isolation rearing can affect various measures of attentional processing. These are reviewed in detail in the introduction to Chapter 3 of this thesis (section 3.1)

1.5.5 Limitations of current animal models

Making progress in the study of neuropsychiatric disorders is impeded by the complex neurobiology of higher brain functions involved in these diseases, as well as

the ethical and practical issues with examining the living human brain (Nestler and Hyman, 2010). Animal models thus provide an invaluable service in this respect. There are, however, a number of limitations to current animal models.

Many models seek to mimic the behavioural symptoms of the disorder (face validity) and then investigate the corresponding neurological changes. Still others take the opposite approach by attempting to alter brain systems thought to be affected in the disorder (construct validity) in the attempt to produce a behavioural phenotype similar to that seen in the clinical population. The issue with these approaches is that (as will be discussed later in this thesis) when dealing with complex behaviours such as those affected in these disorders, similar behavioural effects are not necessarily produced by the same neurological alterations. Our knowledge of the mechanistic basis for schizophrenia is limited; so is our ability to create animal models that mimic these mechanisms. Current models are often mechanistically speculative. Additionally, it is unlikely that the vast and variable behavioural phenotype produced in a disorder such as schizophrenia involves a single neurotransmitter system, or is due to changes in a single brain area. This is one possible explanation for why current animal models have shown poor predictive validity for drug efficacy in human disease (Markou *et al*, 2009).

Issues of etiological validity are highlighted in many current animal models. While some models, particularly those in the developmental/environmental category, are able to mimic some aspects of the etiology of the disorder they are attempting to model, others do not. For example, while a dose of amphetamine may produce symptoms of schizophrenia in rodents, and the DA system is likely implicated in psychosis, this is not how schizophrenia develops in the clinical population. Furthermore, all of the models discussed above (sections 1.5.1-1.5.4) focus on only one intervention (genetic,

environmental, etc) while it is currently believed that diseases like schizophrenia are actually caused by multiple factors working together to produce the disorder.

1.6 The two-hit approach to modeling neuropsychiatric disorders

As our understanding of neurodevelopmental disorders advances, so does our ability to create better animal models. As discussed in section 1.2.3, schizophrenia is considered to be a neurodevelopmental disorder caused by some combination of genetic and environmental factors. In the past, efforts to model schizophrenia in animals have concentrated on using only one experimental intervention to attempt to produce disease characteristics in the model. Recently, attention has turned to the possibility of developing animal models that incorporate what has come to be referred to as a "two-hit" approach. In doing so, researchers are able to create models that better mimic the etiology of the disorder. In animal models of schizophrenia, this usually takes the form of using two of the already established interventions described in section 1.5. The two manipulations may be from different categories of models (e.g. gene-environment models) or may be from the same model category (e.g. two developmentally based interventions). Established models that have been used in combination include maternal infection (Dalton *et al*, 2012; Deslauriers *et al*, 2013) and stress paradigms (Deslauriers *et al*, 2013), as well as a variety of gene-environment models and a combination of NMDA receptor antagonism and social isolation rearing.

1.6.1 Gene-environment interaction models

As schizophrenia is currently believed to be caused by some combination of genetic and environmental influences, it is no surprise that some of the first two-hit

animal models of the disorder implicated both a genetic manipulation and an environmental insult. A number of DISC1 transgenic mouse models (which display phenotypes relevant to both schizophrenia and depression) have been paired with both early immune action models and later life stress models (social isolation or social defeat). Results have shown changes in locomotion, PPI, LI, memory, as well as changes in measures of depression and anxiety, with many of the findings being the result of gene x environment interactions specifically (results reviewed in Cash-Padgett and Jaaro-Peled, 2013). Gene-environment models have also been investigated using the NRG1 mutant mouse model combined with various stress paradigms, cannabis exposure, and housing conditions (Karl, 2013). While the obvious advantage of such models is that they aim to mimic the current gene x environment theory of schizophrenia development, most candidate genes have only a loose connection with the disorder, many have not succeeded in producing behavioural symptoms and fewer still have been assessed for predictive validity.

1.6.2 Two-hit models using social isolation rearing

Social isolation rearing has been used a number of times in studies of potential two-hit animal models of schizophrenia. The majority of those studies combined it with NMDA receptor antagonism. A study by Ashby *et al*, (2010) assessed the effects of combined NMDA receptor antagonism via MK-801 (0.5mg/kg i.p 2x/day for 7 days from PND 56-62) and social isolation rearing (rats housed in isolation or in groups of 4 from PND 21 onward) on locomotor behavior and hippocampal long term potentiation (LTP). Interestingly, while they found that both interventions produced effects on one or

both measures of interest, the combination of the two did not produce any detectable additive or synergistic effects.

Another study which combined MK-801 treatment (0.5mg/kg i.p 2x/day for 7 days from PND 56-62) with social isolation rearing (rats housed in isolation or in groups of 4 on PND21) on male rats was conducted by Hickey *et al*, (2012). While the results indicated that the experimental manipulations produced significant changes in locomotor activity, GAT-1 activity and $GABA_A$ receptor expression, they found no indication that the double hit produced any combined effects.

In contrast, work by Lim *et al*, (2012) also used MK-801 treatment and social isolation rearing (rats housed alone or in groups of 4-5 from PND 21 onward), but administered the MK-801 during the post-natal period from PND 7-10 (0.2 mg/kg i.p. 1x/day for 4 days) with behavioural testing occurring in adulthood. While this study found PPI deficits in all animals that were housed in isolation, the effects were more robust in those rats who also received MK-801. Additionally only the "two-hit rats" showed hyperlocomotion and impaired memory, indicating that MK-801 and social isolation rearing together produce greater effect than did either alone.

The results of these studies and others with similar results (Gaskin *et al*, 2014; Gilabert-Juan *et al*, 2013; Hawken *et al*, 2013) indicate that MK-801 treatment and social isolation rearing may have potential as a two-hit animals model of schizophrenia but it may depend on the MK-801 treatment paradigm used.

1.6.3 Neonatal DOM treatment and social isolation rearing as a potential 2-hit model

As discussed in the previous section, a number of studies have combined the social isolation rearing model with a pharmacological model in an attempt to produce a

two-hit animal model of schizophrenia. The presence or lack of an additive effect in these studies appears to be the result of variations in the timing of the MK-801 treatment (postnatal vs. adolescence) indicating that social isolation rearing does show promise as one of the experimental manipulations of a two-hit animal model. To-date neonatal DOM treatment has never been combined with any other model in an effort to produce a two-hit model but has the potential to easily be combined with social isolation rearing in order to investigate the further potential of these models, both alone and in combination.

Because both of these models produce behavioural and neurological changes in a progressive developmental manner, a model which combines both will follow the origins of diseases like schizophrenia, as well as increase our understanding of how these diseases develop. Additionally, a model that combines two or more interventions to produce changes is further in line with the current understanding of how neuropsychiatric disorders like schizophrenia develop. Both neonatal low-dose DOM treatment and social isolation rearing have modeled a number of behavioural and neurological characteristics of neuropsychiatric disorders individually. It is therefore possible that when combined, these early life manipulations will lead to a stronger and more extensive behavioural phenotype. While the possibility exists that a new model may be created that provides a basis for the testing of new pharmacological treatments, the immediate outcome of studying the combination of these two models is the potential to learn more about how a variety of factors may contribute to the behavioural and neurological changes seen in neuropsychiatric disorders, as well as to increase our understanding of how different experimental manipulations work together to produce the variety of changes seen in animal models.

1.7 Summary

1.7.1 Statement of the problem

Neuropsychiatric disorders affect a significant portion of society and as a class of diseases, are often poorly understood and difficult to treat. These disorders are a serious burden to the economy, to society and to the families and people they affect. While treatments do currently exist, they are far from ideal and a diagnosis of a disorder such as schizophrenia is likely to lead to a significant decline in functioning and quality of life.

Attentional processing is a higher order cognitive behaviour which is fundamental to many important brain processes and is found to be affected in a number of human neuropsychiatric disorders. It is believed that alterations in brain functioning during critical periods of nervous system development may be a contributing factor to such disorders. To date, research in our lab has revealed that chronic administration of low, sub-convulsive doses of DOM during a critical period of brain development can cause permanent alterations in behaviour and brain physiology in adulthood. Many of these subtle cognitive changes could be interpreted in the context of alterations to attentional processing, highlighting the potential usefulness of neonatal DOM treatment in modeling the attentional deficits present in many illnesses.

1.7.2 Experimental purpose, hypotheses and objectives

The purpose of the experiments described herein was to investigate the role of chemically (DOM) and environmentally (isolation rearing) induced changes in brain development on both relevant behavioural testing paradigms and region-specific expression of proteins previously associated with these behaviours. In doing so, the

intent was to use these experimental manipulations to better understand how early developmental alterations can have a long lasting effect on the CNS and how such alterations may contribute to the development of neuropsychiatric disorders, either alone or in combination as a novel approach to investigating the "two-hit" hypothesis of psychiatric disease.

The specific hypotheses tested were:

1) That both neonatal low-dose DOM treatment during the second week of postnatal life and social isolation rearing from weaning would lead to alterations in behavioural measures of attentional processing in adult rats.

2) That the same experimental treatments would result in changes in protein expression relevant to the neurotransmitter systems previously implicated in those behaviours.

3) That neonatal low-dose DOM treatment during the second week of postnatal life and social isolation rearing from weaning combined would lead to more dramatic and consistent behavioural and neurochemical changes than either intervention alone.

To test these hypotheses, the thesis had 3 primary objectives as follows:

1) To investigate the effect of neonatal low-dose DOM treatment and social isolation rearing on behavioural measures of attentional processing.

2) To explore the effects of neonatal low-dose DOM treatment and social isolation rearing on protein expression in the brain in systems known to be implicated in attentional processing.

3) To assess the interaction between neonatal DOM and social isolation rearing in rats and thereby better model aspects of human neuropsychiatric disorders.

1.8 References

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Chapter 2

Development of protocols to assess different aspects of latent inhibition behaviour in adult, untreated rats using a conditioned emotional response task

Summary

Latent inhibition is a normal behavioural process that occurs when previous non-reinforced exposure to a stimulus impairs the ability of that stimulus to subsequently enter into new associations. Both disrupted and abnormally persistent LI are believed to illustrate different aspects of impaired attentional processing and potentially different categories of symptoms in diseases like schizophrenia. In order to test for both forms of impaired attentional processing in studies described later in this thesis, it was first necessary to create paradigms to produce both LI and a lack of LI in adult, control animals. Therefore, the purpose of this study was to create and assess two different protocols for testing LI in adult rats.

2.1 Introduction

Latent inhibition is a normal cognitive process whereby previous non-reinforced experience with a particular stimulus impairs the ability of that stimulus to subsequently enter into new associations. According to Lubow (1989), who first proposed the conditioned attention theory, when a CS is followed by no consequence, the animal learns to ignore that stimulus. As a result, during later pairing of the CS with a US the animal fails to attend to the CS and associative learning is impaired. This view of LI as learned inattention presents it as an adaptive mechanism, important for the proper processing of incoming stimuli, as normally the ability to ignore a stimulus that was irrelevant in the past would be beneficial (Lubow, 1997). Observed across many different species, including rats and humans (Lubow and Gewirtz, 1995), LI is reliably disrupted in humans with schizophrenia (Baruch *et al*, 1988; Gal *et al*, 2005, 2009; Lubow and Gewirtz, 1995; McDowd *et al*, 1993) and has become widely used in studies of the neural alterations of various psychiatric disorders (Bitanihirwe *et al*, 2010; Enomoto *et al*, 2011; Solomon *et al*, 1981; Weiner and Arad, 2009; Weiner and Feldon, 1987), as well as in the search for useful animal models of such disorders (Lubow, 1989, 2005; Moser *et al*, 2000; Zuckerman *et al*, 2003).

More recently it has been suggested that different aspects of the observed changes in LI in the clinical population, and within potential animal models, might illustrate different aspects and symptom categories of schizophrenia (Weiner and Arad, 2009; Weiner, 2003). According to this theory there are 2 ways of looking at changes in LI behaviour, namely, the disruption of LI versus the abnormal persistence of LI. Disruption of normal LI in experimental animals occurs when a control group displays LI while the experimental group does not. Abnormally persistent LI in experimental

animals is observed when the control group does not display LI, but the experimental group does (Weiner, 2003). See section 1.3.4.1 for an theoretical explanation of the brain systems involved in LI.

According to this theory, dysfunctional attentional control can, therefore, be exhibited by these two opposing forms of LI behaviour. In the first case, disrupted LI is caused by a failure to inhibit attention to irrelevant stimuli. This behaviour would theoretically result in abnormally increased perception of salience and distractibility, potentially leading to psychotic symptoms such as those seen in the positive symptom category of schizophrenia. In the second case, abnormally persistent LI is caused by a failure to re-deploy attention when previously irrelevant stimuli become relevant again. This results in cognitive inflexibility and impairment in attentional shifting which are associated with the negative and cognitive symptoms of schizophrenia (Weiner and Arad, 2009).

In order to fully assess potential changes in LI following experimental manipulations in animals (such as the studies described in subsequent chapters of this thesis), it is necessary to test for both the disruption of LI as well as the abnormal persistence of LI. Thus, it was first necessary to create LI protocols that would produce both a normal LI effect, and a lack of an LI effect in control animals. As discussed in Chapter 1, LI can be measured using a variety of paradigms. For the purpose of the current experiment, a conditioned emotional response task was chosen because its use in LI testing has been well document and it is possible to easily alter various aspect of the protocol to produce the desired behaviour in control animals (see Lubow, 1989 for review).

2.2 Materials and methods

2.2.1 *Experimental animals*

A total of 52 adult Sprague-Dawley rats were obtained from Charles River Laboratories (PQ, Canada). At the beginning of experimentation, male rats (n = 26) weighed approximately 350g, while female rats (n = 26) weighed approximately 250g. Upon arrival, all animals were single housed on a reversed light-dark cycle (lights on at 7:00pm and off at 7:00am). All rats had *ad libitum* access to food and water (except during testing as outlined below), as well as an opaque PVC tube for environmental enrichment. Rats were left undisturbed for one week before behavioural testing began. Testing was conducted during the dark phase of the light/dark cycle using red lighting to maintain the dark environment. Animals were randomly allocated to either Experiment 1 or Experiment 2 (n = 26 in each) and subsequently assigned to either a pre-exposure (PE) or non pre-exposure (NPE) group with both sexes equally represented (n = 6/7 rats per group), see Figure 2.1. All procedures were conducted according to the guidelines established by the Canadian Council on Animal Care and were approved in advance by the Animal Care Committee at the University of Prince Edward Island.

2.2.2 Latent inhibition testing procedure

Latent inhibition was assessed using a CER task that measured lick suppression behaviour, adapted from Weiner and Arad, (2009). Testing was conducted using a standard rat operant chamber (Med-Associates, VT, USA) equipped with a grid floor, tone-generating speaker and a drinking tube equipped with a lickometer. Experiment 1 was intended to produce the normal LI effect in the animals, while Experiment 2 was intended to produce a disruption to the LI effect. The paradigm was similar for both

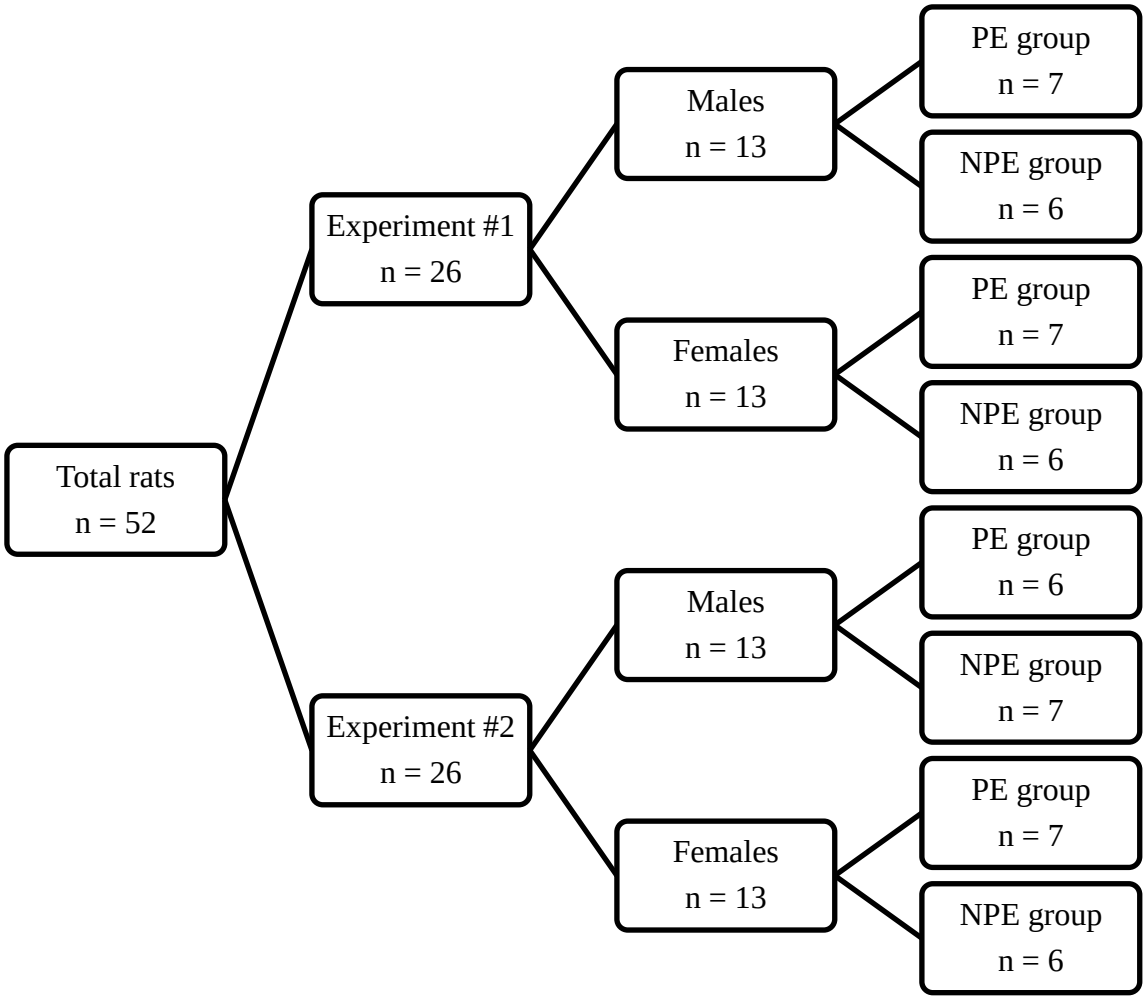

Figure 2.1 The division of experimental animals into the various groups needed for LI testing. PE signifies animals in the pre-exposure group, NPE signifies animals in the non pre-exposure group.

experiments with only the number of tone-shock exposures (the conditioning phase) altered in an attempt to produce the different effects. The testing protocols are described below and summarized in Figure 2.2.

Acclimation to handling (days 1-3): Rats were handled individually for 2 minutes each day. Handling consisted of touching and stroking the rats as they moved freely about the homecage (days 1 and 2), picking up and holding the rats (days 2 and 3) as well as stroking and manipulating the rats while they were being held (day 3).

Water restriction (day 3): All animals were placed on a 23 hour water restriction schedule beginning at the end of day 3. During all subsequent days of the test protocol rats received water for 1 hour each day in their home cages following testing, in addition to having access to water during some components of the test procedure. This water restriction schedule was modeled after the 23-hour water restriction commonly used in similar LI testing paradigms (Almey *et al*, 2013; Lehmann *et al*, 2000; Weiner and Feldon, 1987). The amount of water consumed during these times was recorded. This water restriction schedule was continued throughout the length of the experiment.

Lick training (days 4-8): For 5 days, animals were placed individually into the operant chamber and allowed to drink for 20 minutes. The latency (in seconds) to first lick and the total number of licks were recorded.

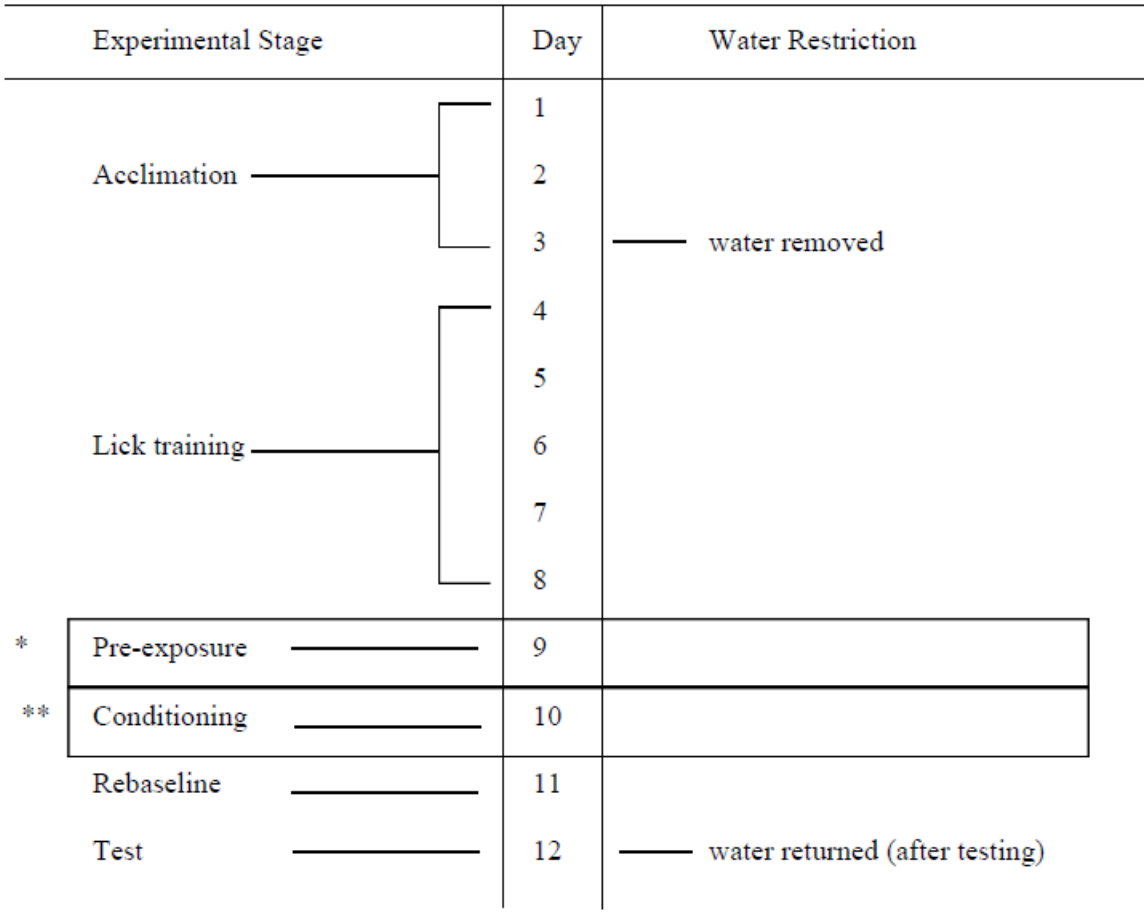

Figure 2.2. Latent inhibition testing protocol

Note: Both the PE and NPE groups received the same treatment at all experimental stages except for the pre-exposure stage on Day 9 as indicated by *. On this day the animals in the PE group received 40 exposures to a 10 second, 80 decibel tone stimulus at one minute intervals, while animals in the NPE group were placed in the operant chamber for an equal amount of time but were not exposed to the tone stimulus.

Testing procedures were identical for Experiments 1 and 2, except for Day 10, as indicated by **. On this day, animals in Experiment 1 received 2 tone-shock pairings within the 15 minute trial, while animals in Experiment 2 received 5 tone-shock pairings, also within 15 minutes.

Pre-exposure (day 9): During the pre-exposure phase, animals were placed into the operant chamber with the drinking tube removed. Animals in the PE group received 40 exposures to a 10 second, 80 decibel (dB) tone stimulus at one minute intervals. Rats in NPE group were placed into the operant chamber for an equal amount of time, but were not exposed to the tone stimulus.

Conditioning (day 10): All rats were placed in the operant chamber with the drinking tube removed. Rats in Experiment 1 received 2 pairings of a 10 second tone immediately followed by a 1 second, 0.5 milliamp (mA) foot shock. Rats in Experiment 2 received 5 pairings of the same intensity tone + foot shock. The tone-shock pairings were distributed at 5 minute intervals (Experiment 1) or 3 minute intervals (Experiment 2) so that the conditioning phase for all rats was a total of 15 minutes.

Rebaseline (day 11): All rats were given a 20 minute drinking session, identical to that of the initial lick training phase. No tone or foot shock was administered. Latency (in seconds) to first lick and total number of licks were recorded.

Test (day 12): All animals were placed in the operant chamber with the drinking tube exposed and were allowed to drink *ad libitum*. Immediately after the 100th lick was recorded, the tone (80 dB) commenced and continued until the 120th lick was recorded or 300 seconds had elapsed, whichever came first. The time to complete licks 80–100 (A) and licks 100–120 (B) was recorded and used to calculate the lick suppression ratio using the formula A/(A+B). Consequently, a value of 0.003 indicates maximum drinking suppression during tone presentation and a score of 0.5 indicates no drinking

suppression. Following testing animals were replaced in their homecages and returned to normal housing conditions.

2.2.3 Data analysis

Independent samples t-tests (1-tailed) were used for data analysis (SPSS Version 19) with Levene's test for equality of variance applied where appropriate. For all analyses $p < 0.05$ indicated a significant difference. Data for males and females was analyzed separately based on previous research and based on the intended use of the protocols established in this study. Sex differences in LI behaviour have been well documented in the clinical populations (Lubow *et al*, 2001), in healthy test subjects (Klosterhalfen *et al*, 2005), and in various animal models (Bethus *et al*, 2005). Additionally, previous studies using neonatal low-dose DOM administration have identified extensive sex difference within the model (Adams *et al*, 2008, 2009; Burt *et al*, 2008a, 2008b; Doucette *et al*, 2003, 2007; Gill *et al*, 2010, 2012; Marriott *et al*, 2012; Perry *et al*, 2009; Robbins *et al*, 2013; Ryan *et al*, 2011). As the purpose of the study described in this chapter was to develop experimental paradigms to be used in testing the neonatal domoate model and as the intention was to investigate sex differences in those studies by analyzing data from males and females separately, it was necessary to assess LI behavior in both sexes in this study. Values in text are expressed as Mean ± standard error of the mean (SEM).

2.3 Results

2.3.1 Experiment 1

2.3.1.1 Licking behaviour during training

Analysis of the average number of licks each animal made while in the operant chamber during the 5 days of lick training showed no significant difference between females in the PE group and those in the NPE group [$t_{11} = 0.349$, $p = 0.734$]. However in males, a significant difference between groups was found [$t_{11} = 2.878$, $p = 0.015$] with males in the PE group (2696.1 ± 273.43) licking on average more than those in the NPE group (1745.5 ± 156.83). Analysis of the latency to begin drinking after being placed in the operant chamber revealed no significant differences between PE and NPE groups in either the male or female animals.

2.3.1.2 Licking behaviour during rebaseline

As with the licking behaviour observed during lick training, analysis of the number of licks taken by the rats during the single rebaseline day revealed that, in females, no significant difference was seen between those animals in the PE group and those in the NPE group [$t_{11} = -0.484$, $p = 0.638$]. In males, however, a significant difference between groups was again found [$t_{11} = 2.364$, $p = 0.038$] with males in the PE group (3772.0 ± 340.47) licking on average more than those in the NPE group (2661.5 ± 314.7). With respect to the latency to begin drinking in the Rebaseline phase, no differences in drinking behaviour were observed between those animals in the PE groups versus the NPE groups in either sex.

2.3.1.3 Drinking behaviour in the homecage

T-tests were conducted to investigate the amount of water consumed by the rats in the 1 hour period when they were returned to their home cages following each day of the testing protocol. In both males and females, no significant differences between PE and NPE groups were observed in the amount of water consumed once they were returned to their homecages. See Table 2.1 for a review of these findings.

2.3.1.4 Latent inhibition as measured by lick suppression

In males it was found that rats in the PE group (0.48 ± 0.091) displayed less lick suppression than did those in the NPE group (0.04 ± 0.012) [t_{11} = 4.451, p = 0.001], thereby exhibiting a significant LI effect (Figure 2.3A). Although females displayed the same general trend of less lick suppression in the PE group (0.20 ± 0.086) versus the NPE group (0.11 ± 0.050), the difference was not statistically significant [t_{11} = 0.920, p = 0.377] (Figure 2.3B).

2.3.2 Experiment 2

2.3.2.1 Licking behaviour during training

An analysis of the average number of licks each animal made while in the operant chamber during the 5 days of lick training showed that no significant differences in average number of licks between PE and NPE animals were observed in either males or females. In the analysis of latency to begin drinking after being placed in the chamber, no significant difference was seen between PE and NPE groups in neither male nor female animals.

Table 2.1 Mean ± SEM amount of water (mL) consumed by rats in each group during the one hour period after being returned to the homecage following testing. LT designates Lick Training. [a,b] indicates $p < 0.05$ for males versus females in the PE and NPE conditions respectively, within each row.

	Male		Female	
Stage	PE	NPE	PE	NPE
Avg LT days	14.29 ± 0.988 [a]	13.63 ± 0.698 [a]	10.42 ± 0.681 [b]	10.70 ± 0.882 [b]
Pre-exposure	25.73 ± 1.399 [a]	22.65 ± 1.435 [a]	18.87 ± 2.149 [b]	15.33 ± 3.048 [b]
Conditioning	24.71 ± 1.175 [a]	24.82 ± 2.022 [a]	18.27 ± 1.329 [b]	19.67 ± 1.382 [b]
Rebaseline	13.73 ± 0.509 [a]	14.12 ± 1.099 [a]	9.76 ± 1.136 [b]	9.47 ± 2.093 [b]

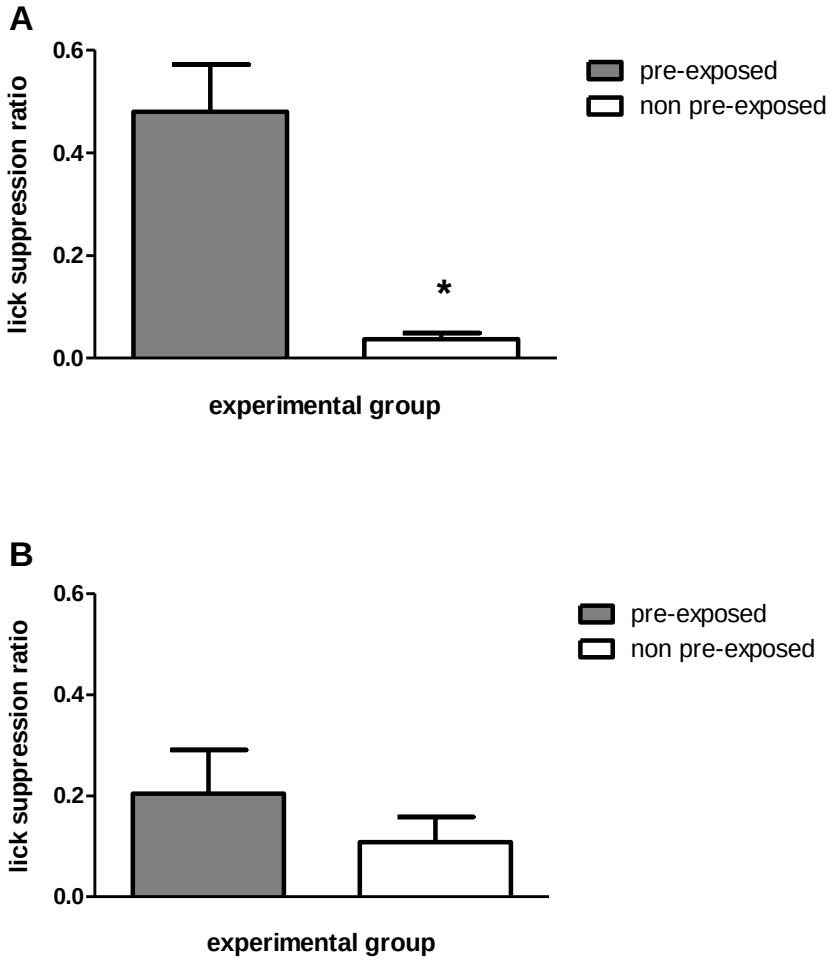

Figure 2.3 Mean lick suppression ratios for adult male (A) and female (B) rats when tested in a paradigm designed to produce normal LI in untreated animals (2 tone-shock pairings during the conditioning phase). Values represent Mean ± SEM. * indicates a significant difference between PE and NPE groups ($p < 0.05$).

2.3.2.2 Licking behaviour during rebaseline [a]

When analyzing the number of licks taken on the rebaseline day, no significant differences between PE and NPE groups were observed in either males or females. An analysis of the latency to complete the first lick revealed no significant differences between PE and NPE groups in males or females.

2.3.2.3 Drinking behaviour in the homecage

As in Experiment 1, an analysis of the amount of water consumed after being returned to their homecages after testing revealed no group differences were observed between PE and NPE animals in either sex. These findings continued throughout testing with similar results being seen on conditioning, pre-exposure and rebaseline days (see Table 2.2).

2.3.2.4 Latent inhibition as measured by lick suppression

No significant differences in lick suppression were observed between PE and NPE groups in the male or female animals (Figure 2.4).

2.4 Discussion

The aim of the present study was to produce the normal LI effect and a lack of the LI effect in adult, untreated animals by altering the testing paradigm. In Experiment 1 the parameters chosen resulted in the production of normal LI in males, as demonstrated by the animals in the NPE group showing significantly greater suppression

[a] Analyses in this section were conducted using data from only half of the animals because of a computer malfunction.

Table 2.2 Mean ± SEM amount of water (mL) consumed by rats in each group during the one hour after being returned to the homecage following testing. LT designates Lick Training. [a,b] indicates $p < 0.05$ for males versus females in the PE and NPE conditions respectively, within each row.

	Male		Female	
Stage	PE	NPE	PE	NPE
Avg LT days	16.66 ± 1.725 [a]	16.74 ± 1.236 [a]	11.43 ± 0.751 [b]	12.66 ± 1.360 [b]
Pre-exposure	27.25 ± 1.094 [a]	26.64 ± 1.977 [a]	17.09 ± 1.886 [b]	21.37 ± 1.383 [b]
Conditioning	25.07 ± 1.181 [a]	29.10 ± 1.724 [a]	20.74 ± 1.886 [b]	19.88 ± 1.548 [b]
Rebaseline	17.13 ± 2.131 [a]	17.33 ± 1.311 [a]	14.07 ± 1.347 [b]	13.42 ± 1.133 [b]

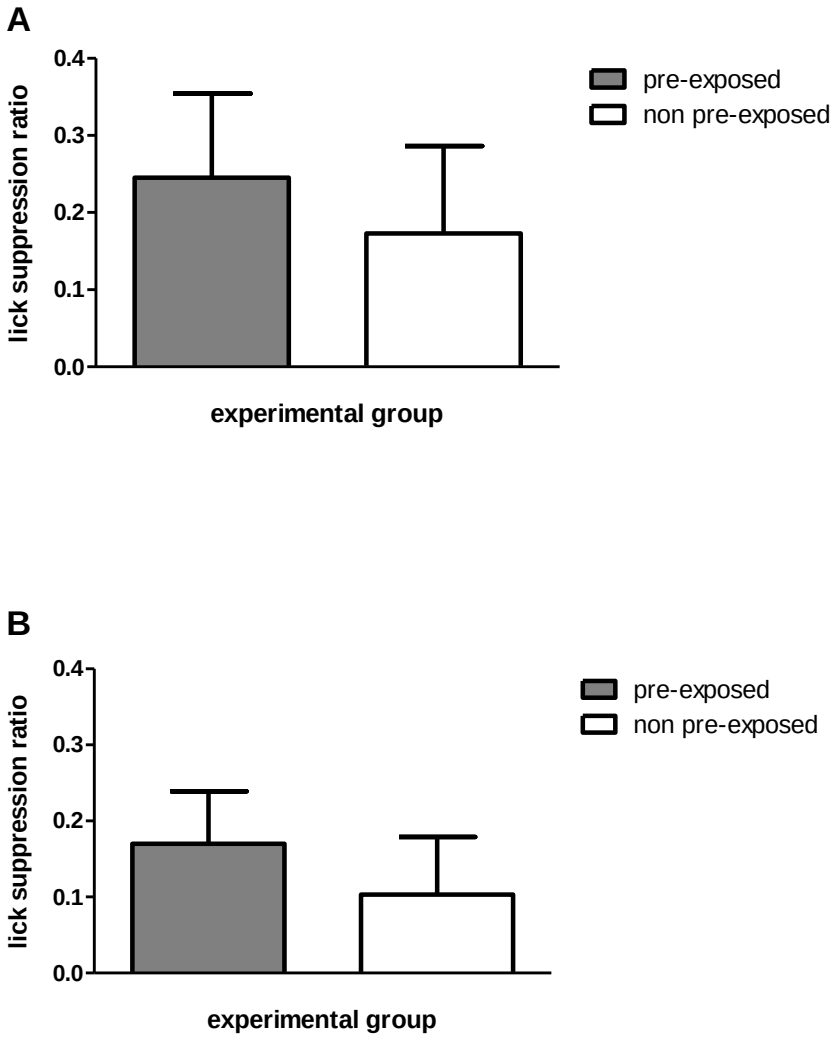

Figure 2.4 Mean lick suppression ratios for adult male (A) and female (B) rats when tested in a paradigm designed to produce a lack of LI in untreated animals (5 tone-shock pairings during the conditioning phase). Values represent Mean ± SEM. * indicates a significant difference between PE and NPE groups ($p < 0.05$).

of drinking behaviour during the tone, compared to those animals in the PE group. According to current theory (Lubow, 1997) this behaviour was produced because the animals in the PE group had been exposed to a prior unreinforced experience with the tone and learned that the tone stimulus was meaningless. Consequently, this prior unreinforced exposure to the tone retarded the leaning of the association between the tone and the shock stimulus during the later conditioning phase. When the tone was then played during testing, the animals did not display the freezing behaviour which would result in lick suppression because they had not made a strong (or potentially any) association between the tone and the shock. The opposite behaviour was seen in the NPE group animals. Rats in the NPE group did not have prior experience with the tone before the conditioning phase. Consequently, these animals presumably made a strong association between the tone and the shock, which caused the rats to display a fear response of freezing/lick suppression on presentation of the tone, believing that the tone predicted the onset of the shock.

It was important to investigate how this behaviour may differ in males and females as sex differences have been previously reported in this model (Adams *et al*, 2008, 2009; Burt *et al*, 2008a, 2008b; Doucette *et al*, 2007; Marriott *et al*, 2012; Robbins *et al*, 2013; Ryan *et al*, 2011). Sex-dependent analysis of the data in Experiment 1 revealed that the LI effect was present in males (Figure 2.3A), but that females only displayed the same general trend (Figure 2.3B) and did not show a statistically significant LI effect.

Experiment 2 evaluated a protocol designed to produce a lack of LI despite the PE group having prior experience with the tone stimulus while the NPE group did not. This was accomplished by maintaining the same pre-exposure procedure as in

Experiment 1 (40 tone exposures for the animals in the PE group; no tone exposures for the animals in the NPE group), but by changing the conditioning phase so that all animals experienced 5 tone-shock pairings instead of 2. As predicted, there was no significant difference in lick suppression during testing between the PE and NPE groups (see section 2.3.2.4). Consistent with Experiment 1, the animals in the PE group received non-reinforced exposure to the tone during the pre-exposure phase, teaching them that the tone was irrelevant. However, the results of Experiment 2 indicate that if the animal receives 5 tone-shock pairings, they are able to make an association between the tone and the shock stimulus regardless of their previous exposure. Presumably this is because the tone has again gained relevance. As before, data from males and females was analysed separately and it was found that neither sex exhibited LI using this paradigm (Figures 2.4A and 2.4B)

The sex differences observed in this study were not entirely surprising considering that the existence of sex differences in LI has been shown in clinical populations (Lubow *et al*, 2001), in healthy test subjects (Klosterhalfen *et al*, 2005), and in various animal models (Bethus *et al*, 2005). The reason(s) for sex differences in LI is/are currently unknown. Higher concentrations of estrogen has been proposed as one reason for the lack of LI in females, but studies examining the effect of estrogen on the LI behaviour of rats have lead to contradictory results. In some studies, the presence of high concentrations of circulating estrogen during the conditioning phase (caused by either being in the proestrus phase of the estrus cycle, or in response to treatment with estradiol in ovariectomized rats) has been shown to lead to disrupted LI (Arad and Weiner, 2008; Nofrey *et al*, 2008; Quinlan *et al*, 2010). Similarly, the presence of low concentrations of circulating estrogen during conditioning reportedly leads to intact LI

(Arad and Weiner, 2008; Nofrey *et al*, 2008; Quinlan *et al*, 2010). Other studies, however, have shown contrasting results, finding that the elimination of circulating estrogen by ovariectomy leads to disrupted LI, and estrogen replacement following ovariectomy eliminates this LI deficit (Arad and Weiner, 2009, 2010a, 2010b). These results suggest that estrogen facilitates selective attention, but the precise role of estrogen in LI behaviour remains unclear.

Another possible explanation for the observed sex differences is the potential for various experimental paradigms to affect the sexes in different ways. As outlined by Lubow (1989), there are many experimental variables that can affect LI. These include the similarity of the pre-exposure stimulus and the conditioning stimulus, the number of stimulus pre-exposures, the duration of the pre-exposed stimulus and conditioned stimulus, the intensity of the pre-exposed stimulus and conditioned stimulus, the inter-stimulus interval, the time between stimulus pre-exposure and conditioning, the time between conditioning and testing phases, as well as many others and among all of these there is the potential for interaction between variables. While the majority of studies involving LI were historically done on males only (both for human and animal studies), many recent studies have included females and have shown that testing paradigms which produce different effects in male and females are not unusual. While some studies have shown males exhibiting LI to a greater degree than females (Lubow *et al*, 2001), other studies have shown a stronger LI effect in females as compared to males (Klosterhalfen *et al*, 2005). Still other studies have found an LI effect only in female controls, but subsequently found LI alterations in both sexes following drug treatment (Wang *et al*, 2012). It therefore appears that while both sexes are capable of displaying LI, the effect can likely be influenced by a multitude of experimental factors. This assertion is

supported by our finding that slight alterations to the paradigm can produce LI or a lack of LI in male animals. Such paradigm-dependant sensitivity may explain the lack of an LI effect in the females of this study.

It appears that the issue with the females was that the animals in the PE group were still making a strong association between the tone and the shock. Despite their previous non-reinforced experience with the tone, they were not showing the learned irrelevance towards that stimulus. Therefore in order to produce an LI effect in females, we would need to alter the paradigm so that the tone is deemed irrelevant. While this could be accomplished in many ways, one option might include increasing the number of exposures to the tone during the pre-exposure phase (e.g. have the tone play 80 times instead of the 40 that were done in this study). By altering the paradigm during the pre-exposure phase, thereby providing additional non-reinforced exposure to the tone, we would theoretically be increasing the likelihood that the tone will be ignored or deemed irrelevant during the conditioning phase. Another option is to alter the paradigm during the conditioning stage by decreasing the number of tone-shock pairings (for example, having only 1 pairing, instead of the 2 that was used in Experiment 1). This change would theoretically also lead to decreased salience of the tone stimulus. Finally, it is possible that some combination of paradigm alterations might be required (e.g. increased experience with the tone during the pre-exposure phase and reduced pairings of the tone with the shock during conditioning). For the purposes of the experiments described in Chapters 3 and 4 of this thesis, however, it was felt a higher priority that all animals experience the same behavioural testing paradigm. Thus, while it would be interesting in the future to determine if different testing procedures are able to be used to produce

identical LI effects in males and females, such protocol variations were not used for the later studies described in this thesis.

The possibility that one of the sexes would show an LI effect while the other did not was not unexpected given past research that has shown LI of varying strengths in one sex vs. the other (Klosterhalfen *et al*, 2005; Lubow *et al*, 2001), or even a finding of LI in only one sex (Kaplan and Lubow, 2011) consistent with the current work. Furthermore it is difficult to indentify precisely how widespread this phenomenon may be given that many studies use exclusively male rats (Alves and Silva, 2001; Ellenbroek *et al*, 1996; Metzger and Riccio, 2009; Sandner *et al*, 2004; Shao *et al*, 2009; Solomon *et al*, 1981; Tenn *et al*, 2005; Weiner *et al*, 1984; Wilkinson *et al*, 1994). Further, a previous study by Wang *et al*, (2012) found a significant LI effect only in female control animals and not in males, but significant LI effects were observed in both sexes following drug treatment.

In conclusion, we were able to produce both LI and a lack of LI in male animals by altering the number of tone-shock pairings used during the conditioning phase of the testing paradigm. Females displayed a lack of LI in both paradigms (Experiment 1 and Experiment 2). While producing the LI effect in females may be possible, it may not be possible to produce a consistent LI effect in both males and females using one experimental paradigm. Given the strong effects found in the males, and given that the purpose of this study was to develop protocols for a larger study where males and females will tested in a consistent manner, it has been concluded that this paradigm will be suitable for the subsequent studies.

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Chapter 3

Chemical and environmental effects on behavioural measures of attentional processing

The data presented in this chapter has been submitted for publication to Neuroscience Letters (January 4 2014, manuscript #NSL-14-17) (latent inhibition results) and will be submitted to Behavioural Brain Research in May 2014 (prepulse inhibition results).

Summary

Deficits in attention have long been identified as a core feature in schizophrenia and related neuropsychiatric disorders. The purpose of this study was to investigate the individual and combined effects of neonatal treatment with DOM and social isolation rearing (both putative animal models of schizophrenia) on LI and PPI; measures of attentional processing. Daily subcutaneous injections of DOM (20 μg/kg) or saline were administered to male and female rat pups from PND 8-14, a time of rapid brain growth and change. After weaning, rats were housed either alone or in groups of 4 until behavioural assessment began on PND 110. When tested for LI, neonatal treatment with DOM abolished LI behaviour in adult male rats regardless of housing condition when tested 48 hours after conditioning, but this effect was not observed in female rats. Social isolation rearing also reduced LI in male rats, but not to the same extent as DOM. When tested again 7 days later, single-housed male rats treated with DOM displayed LI whereas saline treated or group-housed DOM males did not. No significant differences were found in female rats at this time point. When tested for PPI, social isolation resulted in significantly lowered amplitude in male rats while DOM treatment appeared to make animals refractory to the effects of social isolation on PPI amplitude. Social isolation and DOM treatment caused an additive decrease in PPI startle latency that was observed in both male and female rats. We conclude that neonatal DOM and social isolation both impair attentional processing in adult male, and to a lesser extent female rats, although the mechanisms by which this occurs may be different.

3.1 Introduction

Deficits in attention have long been identified as a core feature in schizophrenia and related neuropsychiatric disorders (Anscombe, 1987; Braff, 1993; Nuechterlein and Dawson, 1984). Characterized by impairments in the perception and expression of reality, schizophrenia is a heterogeneous disorder comprised of some combination of positive, negative and cognitive symptoms (American Psychiatry Association, 2013). Positive symptoms refer to abnormal behaviours that have been added to the behavioural repertoire. In patients these symptoms include hallucinations, delusions, psychomotor agitation and disordered thought (Strauss *et al*, 1974). The negative symptoms of schizophrenia are characterized by the absence of normal behaviour and include blunted affect, anhedonia, alogia, and social withdrawal (Strauss *et al*, 1974). Cognitive symptoms include attentional deficits as well as deficits in learning, memory and executive functioning (Gold and Harvey, 1993).

Historically, treatment has focused primarily on the positive symptoms of schizophrenia and these symptoms are generally considered to be the most disruptive. More recently however, it has been suggested that a failure in the ability to reduce the processing of irrelevant incoming information (historically classified as a cognitive symptom) results in such information being afforded undue attention. This improper attentional processing may then result in the development of positive symptoms. It has, therefore, been hypothesized that the positive symptoms of schizophrenia may actually be a consequence of attentional impairments such as those illustrated by disrupted LI and PPI (Schmidt-Hansen and LePelley, 2012).

Latent inhibition is a normal cognitive process whereby previous non-reinforced experience with a particular stimulus impairs the ability of that stimulus to subsequently

enter into new associations. According to Lubow's (1989) conditioned attention theory of LI, when a CS is followed by no consequence, the animal learns to ignore that stimulus. Then, during later pairing of the CS with a US, the animal fails to attend to the CS and associative learning is impaired. This view of LI as learned inattention presents it as an adaptive mechanism, important for the proper processing of incoming stimuli (Lubow, 1997). Normally the ability to ignore a stimulus that was irrelevant in the past would be beneficial, and often even necessary, to proper day-to-day functioning.

It has further been suggested that different aspects of the LI changes observed in both clinical populations and animal models (namely the disruption of LI versus the abnormal persistence of LI) might illustrate different aspects and symptom categories of schizophrenia (Weiner and Arad, 2009; Weiner, 2003). According to this theory, disrupted LI is caused by a failure to inhibit attention to irrelevant stimuli. This behaviour would theoretically result in abnormally increased salience perception and distractibility, potentially leading to psychotic symptoms and therefore the positive symptoms of schizophrenia. However, abnormally persistent LI is caused by a failure to re-deploy attention when previously irrelevant stimuli become relevant again. This indicates cognitive inflexibility and impairment in attentional shifting which are associated with the negative and cognitive symptoms of schizophrenia (Weiner and Arad, 2009).

Another measure of attentional processing is PPI, whereby there is normal suppression of the startle reflex when the startling stimulus is preceded by a less intense, non-startling stimulus (Graham, 1975). This measure of sensory-motor gating is believed to be controlled by structures located in the lower brainstem and mediated by input from the forebrain (Weiss and Feldon, 2001). Observed across many different

species including rats and humans, PPI and LI are reliably disrupted in a variety of neuropsychiatric disorders including schizophrenia (Baruch *et al*, 1988; Braff *et al*, 1978; Kohl *et al*, 2013) and have become widely used in studies of the neural alterations of schizophrenia as well as in the search for useful animal models of the disorder and the preclinical evaluation of potential therapeutic agents (Ellenbroek *et al*, 1996; Koch, 2013; Lubow, 1989, 2005; Moser *et al*, 2000; Zuckerman *et al*, 2003).

Our laboratories at UPEI have previously reported that administration of low, sub-convulsant doses (20 μg/kg) of DOM (a Glu receptor agonist) to neonatal rats during a critical period of CNS development (Dobbing and Smart, 1974), results in later-onset changes in behaviors consistent with both clinical schizophrenia and other animal models of the disorder. Excitatory amino acids, such as Glu, play a critical role in many physiological processes which occur during brain development, making proper Glu signaling necessary for correct maturation of the CNS (McDonald and Johnston, 1990). To date we have reported that DOM administered once daily from PND 8-14 produces altered responses to novelty and reward (Burt *et al*, 2008a, 2008b), changes in cognitive functioning (Adams *et al*, 2009; Doucette *et al*, 2007; Robbins *et al*, 2013), altered social interaction (Ryan *et al*, 2011) and changes in stress response (Gill *et al*, 2012). Many of these changes could be interpreted in the context of alterations to attentional processing. Indeed we have previously reported that both LI (assessed using a CTA paradigm) and PPI are impaired in adult rats following neonatal DOM treatment depending on the sex of the animal and the specific paradigm used (Adams *et al*, 2008a; Marriott *et al*, 2012). In these studies we found that male DOM treated rats show significantly decreased LI when tested 24 hours after conditioning (but not when tested a week later), while female DOM treated rats did not display an LI deficit at 24 hours

post-conditioning, but did show a deficit a week later (Marriott *et al*, 2012). Additionally, DOM treated rats displayed deficits in PPI that were dependent on both sex and time of day (Adams *et al*, 2008a; Marriott *et al*, 2012).

Since Hatch and co-workers (Hatch *et al*, 1963) first reported that housing rats in isolation produced abnormal behavioural reactivity, many studies have shown that rats that experience social isolation (housed one animal per cage for some period of time post-weaning) display a variety of profound behavioural, neurobiological and neuroanatomical differences when compared to those rats who are raised in groups (Ferdman *et al*, 2007; Hall, 1998; Lehmann and Feldon, 2000; Paulus *et al*, 1998; Weiss *et al*, 2004). While many of the changes seen in socially isolated animals are believed to model some symptoms of schizophrenia, the effect of social isolation on LI is poorly understood.

In rodents, disruptions of LI can be achieved using chemical, surgical and environmental interventions including social isolation (for a review of factors affecting LI see Lubow, 1989). Results reported following social isolation, however, indicate that the effects may vary according to the timing of both the isolation and the testing, as well as the rat strain used (Han *et al*, 2012; Shao *et al*, 2009; Weiss *et al*, 2001; Wilkinson *et al*, 1994). Shao *et al*, (2009) reported that male Wistar rats who were housed in isolation for 2 weeks during adolescence (PND 38-51) displayed deficient LI as compared to group housed animals when tested as young adults at PND 66, but not when tested immediately after the isolation period at PND 52. The same investigators later showed that LI was impaired in male Sprague-Dawley rats when they were housed in isolation from PND 21-34 and tested for LI at PND 56 (Han *et al*, 2012). In contrast, Wilkinson *et al*. (1994) examined the effect of 8 weeks of social isolation, both from weaning and

later in adulthood, and found that neither paradigm had any effect on the expression of LI. Further, in a study that looked at the effects of both pre-weaning maternal separation and post-weaning social isolation, Weiss *et al.* (2001) found that while maternal separation affected LI, social isolation conducted post-weaning did not. Of particular relevance to the current study, Feldon *et al*, (1990) looked at the effect of daily handling during the first 3 weeks of life and the effect of being raised in isolation from weaning at PND 21 onward, and found that rats raised in isolation may or may not display altered LI, depending on handling experience. These latter data indicate that the effects of isolation rearing on LI may be subject to interactions with other life experiences and suggest that combining a neonatal insult with an additional insult later in life (a so-called "2-hit" model) may better model neuropsychiatric disorders originating early in life.

In contrast to the differing results reported with LI (above), PPI can be reliably disrupted by post-weaning social isolation (Domeney and Feldon, 1998; Geyer *et al*, 1993; Stevens *et al*, 1997). Consistent with this distinction, a study by Wilkinson *et al.* (1994) found that 8 weeks of post-weaning social isolation resulted in deficits to PPI but not LI. This effect of social isolation on PPI has been shown to be reversible by various antipsychotics (Bakshi *et al*, 1998; Stevens *et al*, 1997; Varty and Higgins, 1995) and is routinely used in preclinical drug development (for reviews see Johansson *et al*, 1995; Swerdlow *et al*, 2008). While housing-mediated disruption of PPI has been produced in a number of studies there is some evidence that the effect can be altered by other experimental factors. Varty *et al*, (1999) demonstrated that rats must be isolated continually for more than 4 weeks after the time of weaning in order to produce the PPI deficits. While this disruption to the PPI behaviour has been shown to persist when tested a second time in experimentally naive rats, exposure to locomotor activity testing

either before or after the initial PPI test abolished the PPI effect in subsequent testing (Domeney and Feldon, 1998). Another study found the isolation rearing induced deficits in PPI were found in rats reared in solid bottomed cages, but not in rats that were raised in cages with wire grid floors (Weiss *et al*, 1999). Finally, it has also been suggested that strain differences may contribute to the presence and strength of the isolation rearing induced disruption of PPI (Varty and Geyer, 1998; Weiss *et al*, 2000).

The study described in this chapter was designed to further investigate the individual and combined effects of neonatal DOM treatment and social isolation rearing on both LI and PPI in male and female rats. In doing this our purpose was to determine the potential usefulness of these paradigms, either alone or together, to model neurodevelopmental disorders like schizophrenia and to gain further understanding of the role of early life events in shaping attentional processing in adulthood.

3.2 Materials and methods

3.2.1 Experimental animals and injection procedure

Experimental animals were born in-house from 10 untimed pregnant Sprague-Dawley rats obtained from Charles River Laboratories (PQ, Canada). The day of parturition was designated PND 0. Within 24 hours of birth, litters were culled to 10-12 pups with an even number of males and females where possible. On PND 7 pups were randomly assigned to either the DOM treatment group or the saline control group and ear-notched for identification purposes. From PND 8-14, pups were weighed and given a single daily subcutaneous (s.c.) injection of 20 µg/kg DOM (BioVectra DCL, PE, Canada) or saline.

On PND 21 rats were weighed, weaned and randomly assigned to either the social isolation housing condition (one rat per cage) or the group housing condition (4 rats per cage), with both sexes and drug groups equally represented in each housing condition. Animals who were group housed were placed with non-littermates of the same sex and drug treatment. All cages were placed in the same colony room so that social isolation housed animals could still see, hear and smell other rats, without having physical contact. This resulted in 4 treatment groups for both males and females: Saline / Group housed (SG), DOM / Group housed (DG), Saline / Single housed (SS) and DOM / Single housed (DS) (see Figure 3.1). All rats (n = 94) received *ad libitum* access to food and water (except during LI testing, as described below) and were left undisturbed until behavioural testing began in adulthood (PND 110). Animals were maintained on a reversed 12:12 hr light-dark cycle (lights off at 7:00am, on at 7:00pm). Testing was conducted during the dark phase of the light/dark cycle using red lighting to maintain the dark environment. All procedures were conducted experimenter blind, and according to the guidelines established by the Canadian Council on Animal Care and were approved by the Animal Care Committee at the University of Prince Edward Island.

3.2.2 Latent inhibition testing procedure

Latent inhibition was measured using a CER task (adapted from Weiner & Arad, 2009) as developed in the study described in Chapter 2. The testing apparatus consisted of a standard rat operant chamber (Med-Associates, VT, USA) with a grid floor, tone-generating speaker and a retractable drinking tube equipped with a lickometer. Prior to testing each animal was randomly assigned to either the PE or NPE group (see Figure 3.1), with both sexes and all treatment groups equally represented (n = 6 animals per

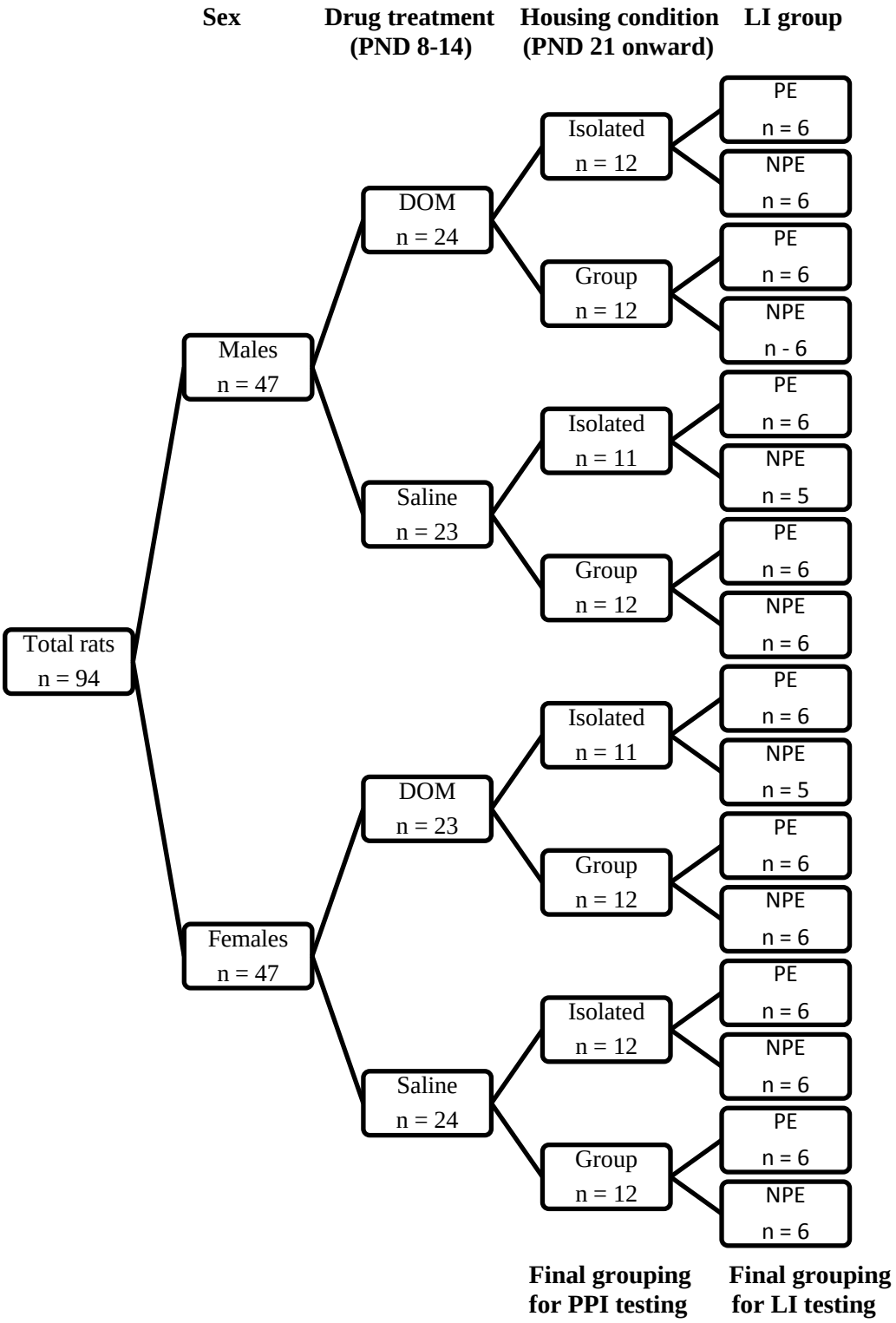

Figure 3.1 The division of experimental animals into the various groups needed for PPI and LI testing. PE signifies animals in the pre-exposure group, NPE signifies animals in the non pre-exposure group.

group). The testing protocol is summarized in Figure 3.2 and consisted of the following components:

Acclimation to handling (days 1-3): Rats were handled individually for 2 minutes each day. Handling consisted of touching and stroking the rats as they moved freely about the homecage (days 1 and 2), picking up and holding the rats (days 2 and 3) as well as stroking and manipulating the rats while they were being held (day 3).

Water restriction (day 3 onwards): All animals were placed on a 23 hour water restriction schedule at the end of day 3. They received water for 1 hour each day in their home cages following testing, in addition to having access to water during some testing phases. The amount of water consumed during these times was recorded. This water restriction schedule was continued throughout the length of the experiment.

Lick training (days 4-8): For 5 days, animals were placed one at a time into the operant chamber and allowed to drink for 20 minutes. Latency to first lick and the total number of licks were recorded.

Pre-exposure (day 9): During the pre-exposure phase, animals were placed into the operant chamber with the drinking tube removed. Animals in the PE group received 40 exposures to a 10 second, 80 dB tone stimulus at 1 minute intervals. Rats in the NPE group were placed into the operant chamber for an equal amount of time, but were not exposed to the tone stimulus.

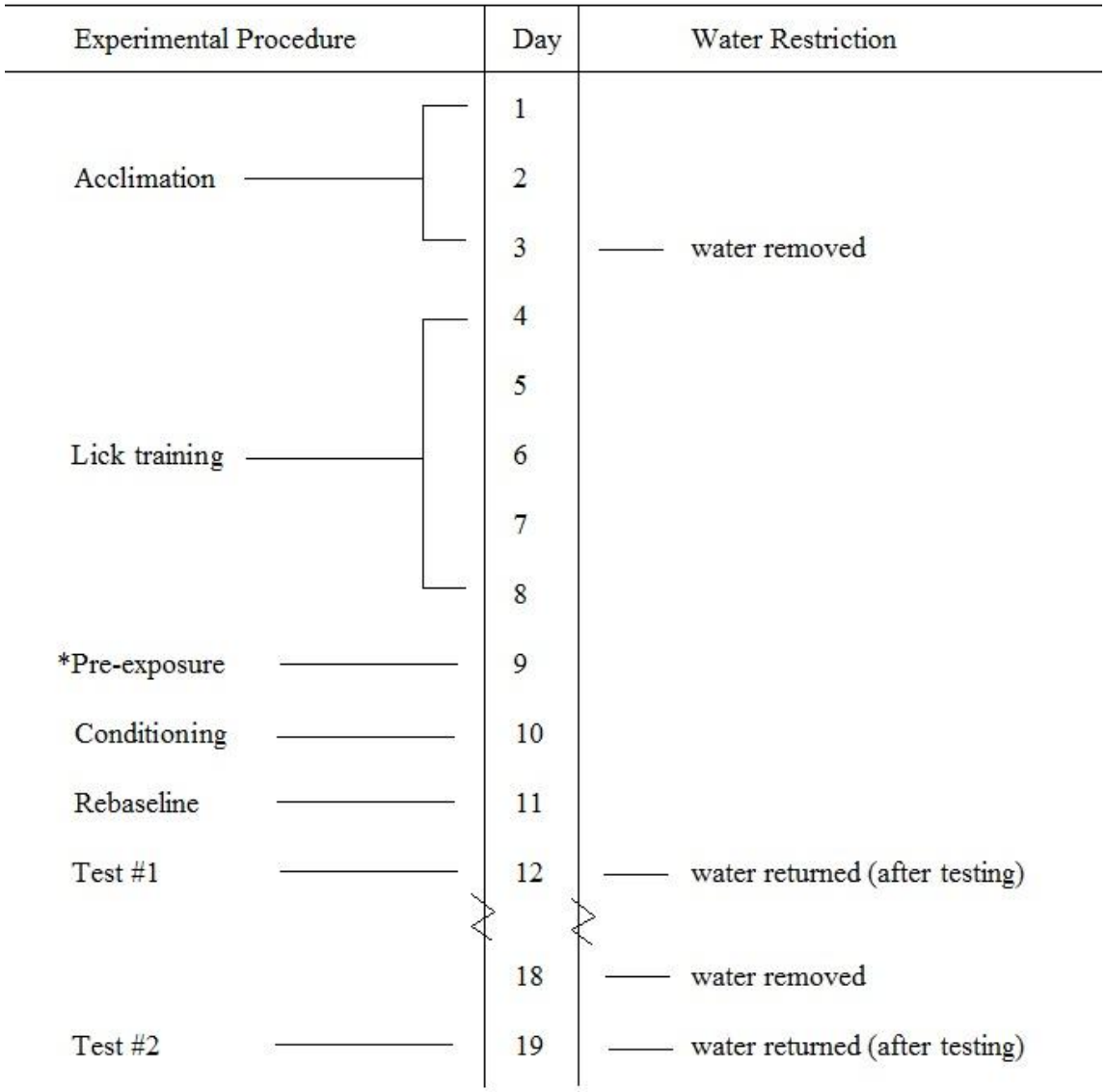

Figure 3.2 Latent inhibition testing protocol

Note: Both the PE and NPE groups received the same treatment at all experimental stages except for the pre-exposure stage on Day 9 as indicated by *. On this day the animals in the PE group received 40 exposures to a 10 second, 80 decibel tone stimulus at one minute intervals, while animals in the NPE group were placed in the operant chamber for an equal amount of time but were not exposed to the tone stimulus.

Conditioning (day 10): All rats were placed in the operant chambers with the drinking tube removed and received 2 pairings of a 10 second tone, immediately followed by a 1 second, 0.5mA foot shock. The tone-shock pairings occurred at minute 5 and minute 10 during the 15 minute trial.

Rebaseline (day 11): All rats were given a 20 minute drinking session, identical to the ones in the initial lick training phase. Latency to first lick and the total number of licks were recorded. No tone or footshock was administered.

Test 1 (day 12): All animals were placed in the operant chamber with the drinking tube exposed and allowed to drink. Immediately after the 100th lick, the tone was switched on. The tone was switched off after the 120th lick or after 300 seconds, whichever happened first. The duration to complete licks 80–100 (A) and licks 100–120 (B) were recorded and used to calculate the lick suppression ratio using the formula A/(A+B). Consequently, a score of 0.003 indicates maximum drinking suppression and a score of 0.5 indicates no drinking suppression. Following testing, animals were returned to their homecages and the water restriction was ended.

Test 2 (one week following Test 1): An additional testing day identical to the first occurred 7 days later (9 days post-conditioning). The animals had their water removed 24 hours before testing and were given free access to water again following testing that same day.

3.2.3 Prepulse inhibition testing procedure

Assessment of PPI began 7 days following the end of LI testing with n = 12 animals per group (see Figure 3.1). All animals were weighed the day before PPI testing began to ensure that they fell within the proper weight range for the apparatus and to check that there were no weight differences between treatment groups. Group differences in weight have the potential to affect the startle measurement as heavier animals produce greater movement (as measured by the accelerometer) than lighter animals.

The startle apparatus was an SR-Lab from San Diego Instruments (CA, USA) and consisted of a clear tube that held the rat over the accelerometer, placed inside a sound attenuating chamber. A background white noise level of 70 dBs was maintained for the duration of testing. All animal received a 5 minute acclimation period to the test chamber before testing began. The experiment consisted of 3 blocks of trials. The intertrial interval averaged 15 seconds (ranging from 10-20 seconds). Measurement of the startle behaviours on all types of trials was obtained by measuring every 1 millisecond (ms) for 100 ms after the onset of the startle pulse. Startle amplitude was calculated as the average of the 100 readings.

Block 1 consisted of 6 white noise startle pulses (40 ms, 120 dBs). This was used to normalize and establish a baseline for each animal's individual startle response. The response to pulse 1 measured initial startle, and the average of pulses 2-6 were used to calculate the baseline startle. The data from these trials was not included in the calculation of the PPI measures.

Block 2 contained 3 types of trials: (1) Startle alone pulses, like those in block 1, (2) no stimulus trials, during which no stimulus other than the background white noise

was administered, and (3) prepulse-pulse trials, which consisted of a 20 ms prepulse (4, 8, 12, or 16 dBs above the background noise) followed 100 ms later (onset to onset) by the startle pulse. Eight trials of each type were administered in random order. Data collected during the no stimulus trials was used to measure the activity of the animals during testing.

Block 3 consisted of 5 final startle pulses identical to those in block 1. This data was used to test for within-test habituation and was not included in the calculation of the PPI behaviours. Additionally, the data from the startle pulse trials (blocks 1 and 3) were used to determine if there was any difference between the two groups in their startle amplitudes, independent of PPI.

Three different prepulse inhibition measures were analyzed. These were (1) the maximum startle amplitude (Vmax), (2) the average startle amplitude (%PPI), and (3) the latency to maximum startle amplitude (Tmax). These are depicted in Figure 3.3. Values for Vmax and %PPI were calculated for each experimental condition by taking the average of the startle alone pulse trials (A) and the prepulse-pulse trials (B) and using the following formula: Vmax or %PPI = 100-(100*(B/A)). Values for Tmax were calculated by taking the average latency for the startle alone pulse trials (A) and the prepulse-pulse trials (B) and using the following formula: Tmax = B-A. See Appendix A for greater detail.

3.2.4 Data analysis

Unless otherwise stated, 3-way ANOVAs (sex x drug treatment x housing condition) were used to analyze weight data, 3-way ANOVAs (drug treatment x housing condition x LI group) were used to analyze the LI data, and 2-way ANOVAs (drug

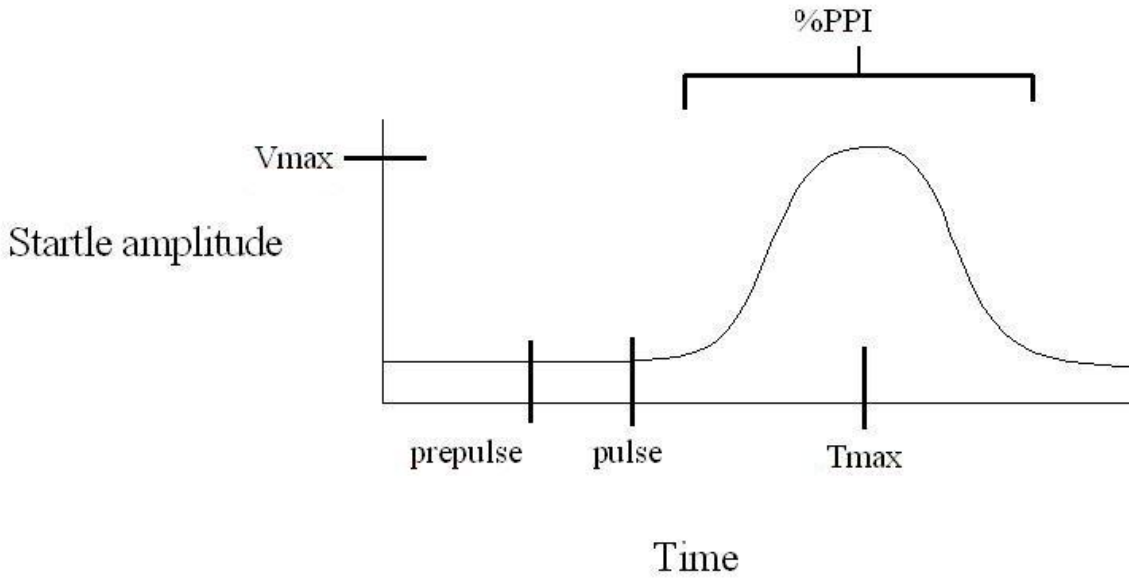

Figure 3.3 A graphical representation of startle amplitude and how each variable was determined. %PPI = Average startle amplitude, Vmax = Maximum startle amplitude, Tmax = Latency to maximum startle amplitude.

treatment X housing condition) were used to analyze the PPI data, with repeated measures (dB level) used where appropriate (SPSS Version 19). Post-hoc comparisons were conducted using Bonferroni t-tests, with Levene's test for equality of variance used where appropriate. A result of $p < 0.05$ indicated significance. Latent inhibition and PPI data for males and females were analyzed separately because sex differences in both measures have been well documented in the clinical populations (Kumari *et al*, 2004; Lubow *et al*, 2001), in healthy test subjects (Klosterhalfen *et al*, 2005), and in various animal models (Bethus *et al*, 2005; Lehmann *et al*, 1999). Additionally, previous studies using neonatal low-dose DOM administration have identified extensive sex difference within the model (Adams *et al*, 2008a, 2009; Burt *et al*, 2008a, 2008b; Doucette *et al*, 2007; Marriott *et al*, 2012; Robbins *et al*, 2013; Ryan *et al*, 2011). Values in text are expressed as Mean ± SEM. Furthermore, the analysis of baseline startle behaviour conducted as a normal part of PPI testing is highly dependent on the weight of the animal, requiring the analysis of each sex separately for those measures.

3.3 Results

3.3.1 Weight

A summary of the recorded weights of all experimental groups can be found in Table 3.1. An analysis of the weights of the rats during the injection protocol (PND 8-14) found a main effect for sex [$F_{1,124} = 4.122$, $p = 0.044$] with males weighing more than females, and a main effect for day [$F_{4.54,562.59} = 2167.898$, $p < 0.001$] with weight increasing with day. No significant effects were observed for drug treatment, nor were any interactions found between any of the variables. When data for males and females were analyzed separately, a main effect for day was observed in both males [$F_{4.33,268.50} =$

Table 3.1 Mean ± SEM for the weight (in grams) of rats during development and testing.

Day	SAL				DOM			
Before weaning	Males (n=32)		Females (n=32)		Males (n=32)		Females (n=32)	
PND 8	18.49 ± 0.318		18.03 ± 0.259		18.68 ± 0.252		18.08 ± 0.273	
PND 9	20.69 ± 0.370		20.24 ± 0.331		20.86 ± 0.291		20.07 ± 0.306	
PND 10	22.94 ± 0.408		22.37 ± 0.306		23.07 ± 0.318		22.23 ± 0.325	
PND 11	25.30 ± 0.409		24.82 ± 0.331		25.48 ± 0.353		24.75 ± 0.324	
PND 12	27.50 ± 0.471		27.09 ± 0.330		27.83 ± 0.378		27.11 ± 0.367	
PND 13	29.95 ± 0.470		29.28 ± 0.344		29.92 ± 0.396		29.34 ± 0.397	
PND 14	32.17 ± 0.495		31.38 ± 0.389		32.20 ± 0.440		31.52 ± 0.424	
	Single housed		Group house		Single house		Group Housed	
After weaning	Males (n=11)	Females (n=12)	Males (n=12)	Females (n=12)	Males (n=12)	Females (n=12)	Males (n=12)	Females (n=12)
PND 21	54.73 ± 1.161	52.08 ± 1.184	53.41 ± 1.612	52.42 ± .996	53.08 ± 1.515	50.67 ± 1.227	53.58 ± 1.221	52.33 ± 1.003
Pre-PPI	686.73 ± 25.795	396.00 ± 19.055	701.83 ± 26.655	369.33 ± 11.922	656.67 ± 16.877	387.91 ± 10.961	678.08 ± 31.120	396.33 ± 19.065

Note: PND signifies the postnatal day on which the measurement was obtained.

1026.880, p <0 0.001] and females [$F_{4.29,266.09}$ = 1148.832, p < 0.001], but no significant differences between experimental groups were observed, nor were any interactions present.

Weights were recorded when animals were weaned on PND21. A main effect for sex was found [$F_{1,87}$ = 4.201, p = 0.043] with males (53.68 ± 0.683g) being heavier than females (51.88 ± 0.787g). No main effects for drug treatment or housing condition were found, nor were any interactions present. When data for males and females was analyzed separately, no significant differences were found.

Animals were weighed again prior to PPI testing. There was a significant main effect for sex [$F_{1,86}$ = 377.519, p < 0.001] with males (680.70 ± 12.636g) weighing more than females (387.38 ± 7.862g). When the data for males and females was analyzed separately, no significant differences were found in any variable, nor were any interactions present.

3.3.2 Latent inhibition

3.3.2.1 Licking behaviour during training

No significant differences in any variable were found in either sex when measuring the average number of licks taken or the latency to begin drinking during training.

3.3.2.2 Licking behaviour during rebaseline

No significant differences in any variable were found in either sex when measuring the average number of licks taken or the latency to begin drinking during rebaseline.

3.3.2.3 Drinking behaviour in the homecage

An analysis of the amount of water consumed by each rat after being returned to the homecage after testing revealed no significant effects in any variable, in either sex, during training days, conditioning days, or rebaseline days. There was, however, a significant effect for housing seen in the males on the pre-exposure day [$F_{1,39} = 5.392$, $p = 0.026$] with group housed animals (33.65 ± 1.112ml) consuming on average more water than single housed animals (30.27 ± 0.834ml). However, this effect was no longer present on subsequent days, and there is, therefore, no reason to believe this would have impacted later testing.

3.3.2.4 Latent inhibition - Test 1 (48 hours after conditioning)

Results obtained in Test 1 (48 hours after conditioning) are summarized in Figure 3.4. An analysis of the data obtained for male rats showed the expected significant main effect for LI group (PE vs. NPE) [$F_{1,39} = 7.098$, $p = 0.011$], with PE rats displaying less suppression of licking (shorter latencies) during the tone than NPE rats (0.173 ± 0.038 and 0.069 ± 0.022, respectively) indicating the presence of LI. The analysis also revealed a significant main effect for drug treatment [$F_{1,39} = 14.424$, $p < 0.001$], with DOM treated rats showing greater lick suppression (0.050 ± 0.011) than saline treated rats (0.196 ± 0.041). A significant interaction between drug treatment and LI group was also observed [$F_{1,39} = 5.241$, $p = 0.028$].

Independent t-tests were used to further investigate the LI effects, as indicated by a significant difference in lick suppression ratios between PE and NPE groups within a particular treatment. In saline treated males, the SG group displayed a strong LI effect [$t_{6.79} = 2.248$, $p = 0.031$] with the PE group (0.250 ± 0.079) showing less lick

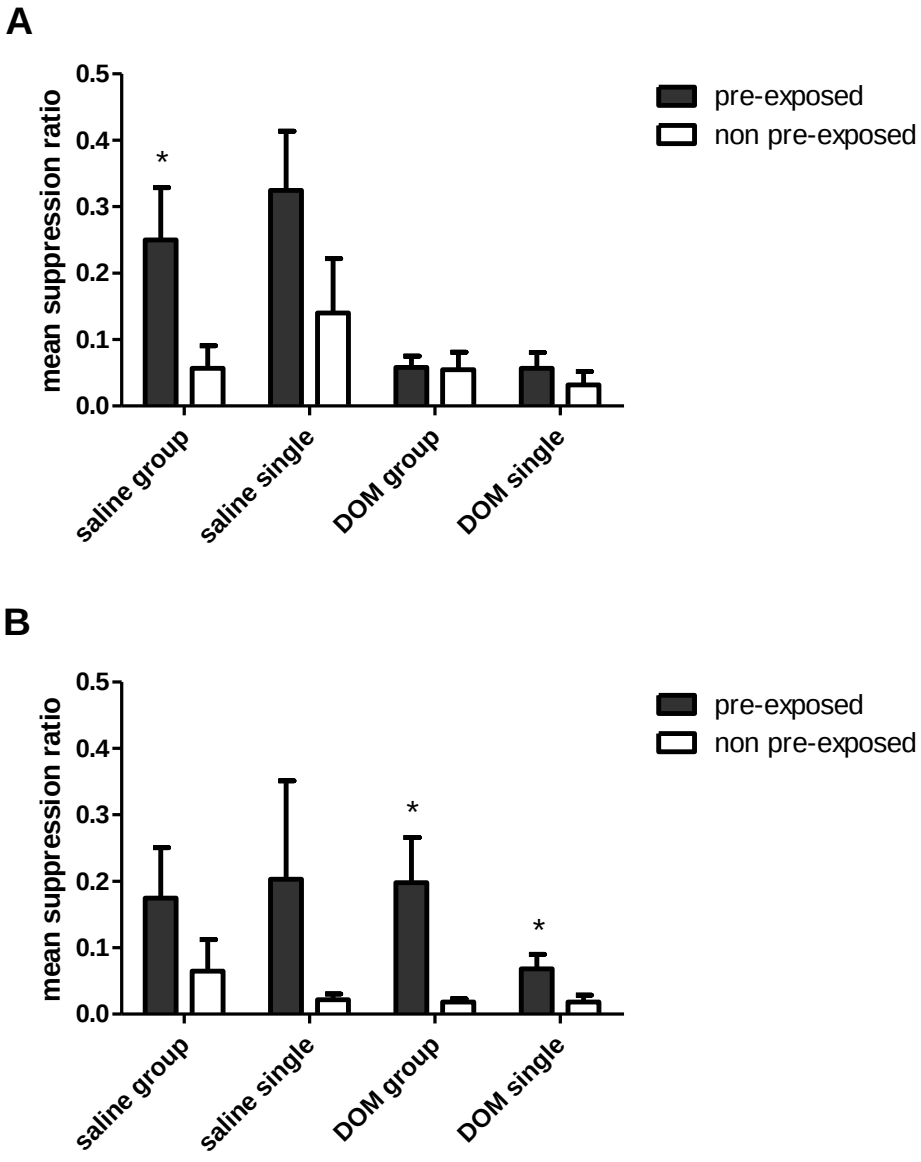

Figure 3.4 Effects of neonatal DOM treatment (20 μg/kg) and housing condition on the expression of LI in a CER paradigm. Lick suppression ratio values are presented for adult male (A) and female (B) rats when tested 48 hours post-conditioning. Values represent Mean ± SEM, asterisk indicates a significant difference between PE and NPE groups, illustrating the presence of LI ($p < 0.05$). n = 5-6 animals per group.

suppression than the NPE group (0.057 ± 0.034). Similarly, although not statistically significant, the SS group showed a marked tendency to display LI (Figure 3.4A). In contrast, the LI effect was completely abolished in all animals that received neonatal DOM treatment regardless of housing condition, with neither the DG group [$t_{10} = 0.108$, $p = 0.459$], nor the DS group [$t_{10} = 0.804$, $p = 0.220$] showing an LI effect (Figure 3.4A). An analysis of the data for female rats also showed a significant main effect for LI group [$F_{1,39} = 7.377$, $p = 0.010$] with PE rats (0.161 ± 0.044) displaying less suppression of licking during the tone than NPE rats (0.031 ± 0.013), but unlike in males no significant effects of drug treatment or drug x PE/NPE group were observed in female rats. Subsequent comparisons between groups using t-tests indicated that in females neither the SG [$t_{10} = 1.236$, $p = 0.123$] nor the SS [$t_{5.03} = 1.223$, $p = 0.138$] group displayed LI, but both of the DOM treated groups did show an LI effect, illustrated by the PE groups having significantly less lick suppression than the NPE groups (DG [$t_{5.05} = 2.656$, $p = 0.023$] and DS [$t_9 = 1.938$, $p = 0.043$]) (Figure 3.4B).

3.3.2.5 Latent inhibition - Test 2 (9 days after conditioning)

When tested again one week later to determine if the effects on LI were persistent, male rats showed a significant main effect for LI group [$F_{1,39} = 4.992$, $p = 0.031$] with animals in the PE group (0.170 ± 0.036) showing less lick suppression than those in the NPE group (0.074 ± 0.020), consistent with the results obtained at 48 hrs. In contrast, the female rats showed neither a significant main effect for LI group nor for drug treatment (though the latter was approaching significance), nor were any statistically significant interactions present.

Independent t-tests were again used to further investigate the LI effects and the data are illustrated in Figure 3.5. In males at this time point, the data obtained for SG and SS groups was suggestive of persistent LI but was not statistically significant [$t_{6.07}$ = 1.245, p = 0.130 and $t_{5.87}$ = 1.747, p = 0.066 for SG and SS, respectively] and as seen at 48 hrs, the DG group showed no evidence of LI [t_{10} = 0.204, p = 0.421]. Surprisingly, however, the DS group displayed a significant LI effect [t_{10} = 1.961, p = 0.039] at this time point (Figure 3.5A) whereas none was seen at 48 hrs (Figure 3.4A). Although significant main effects were absent, data for females were also investigated further using t-tests in order to remain consistent with the analysis of male data, but as expected based on previous analysis, no LI effect was observed in any group (Figure 3.5B).

3.3.3 Prepulse inhibition

3.3.3.1 Analysis of %PPI

Data obtained were analyzed for %PPI (the average of 100 measures, taken every ms for 100 ms after the startling pulse). In male rats, significant main effects were found for dB level [$F_{2.58,110.76}$ = 65.940, p < 0.001], drug treatment [$F_{1,43}$ = 5.329, p = 0.026], and a significant interaction was found between drug treatment and housing condition [$F_{1,43}$ = 4.371, p = 0.042]. When these effects were further investigated using t-tests (Figure 3.6), significant drug effects were found in the single housed males (Figure 3.6C), with SS males displaying significantly lowered %PPI (meaning they displayed on average less inhibition of startle in the presence of a prepulse) as compared to DS males at 74dBs (-1.380 ± 9.643) (20.944 ± 5.360) [t_{21} = 2.070, p = 0.026], 78dBs (7.368 ± 9.138) (37.168 ± 4.086) [t_{21} = 3.066, p = 0.003] and 86dBs (40.385 ± 4.177) (61.840 ± 2.453) [t_{21} = 4.522, p < 0.001]. These drug effects were not seen in the group housed rats

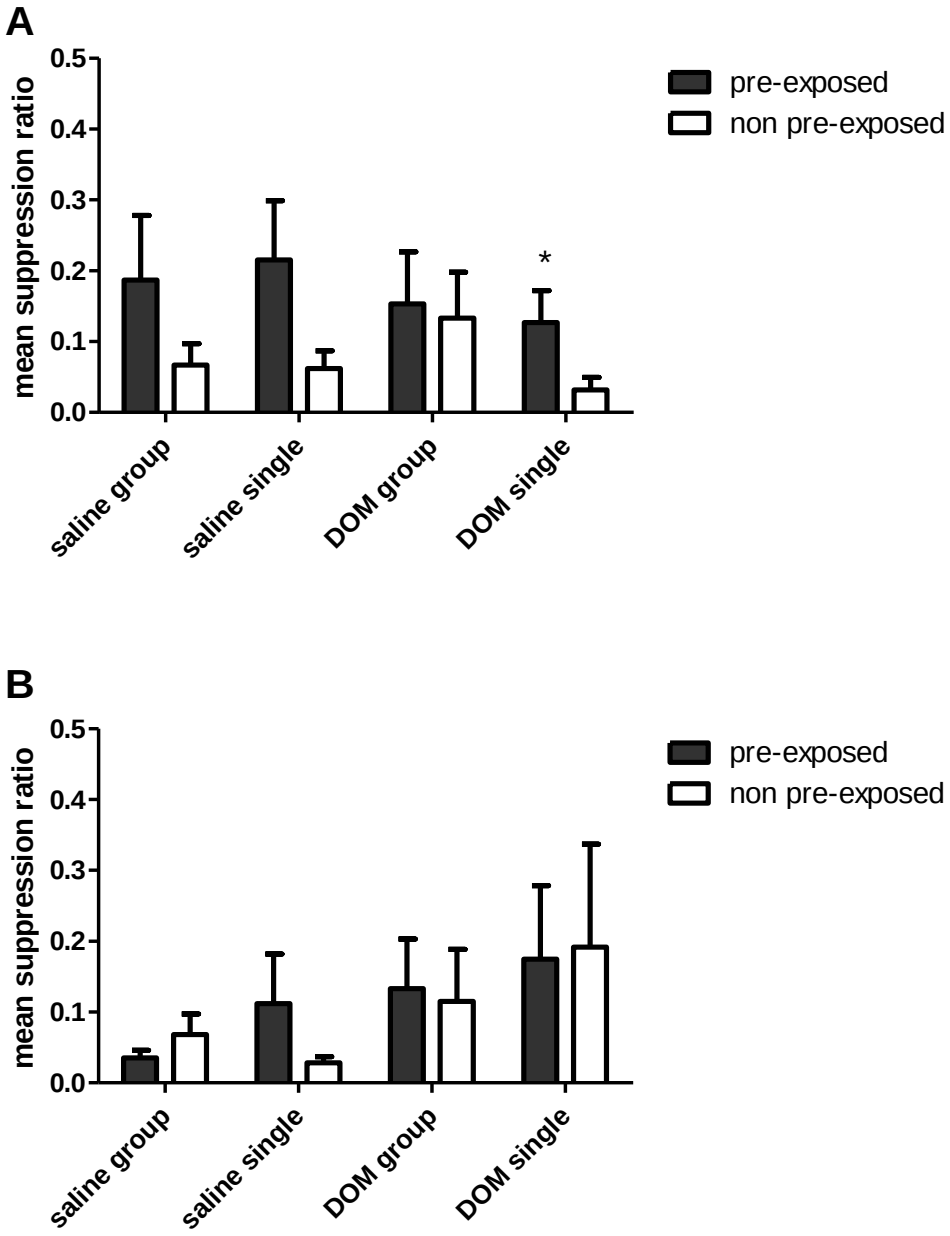

Figure 3.5 Effects of neonatal DOM treatment (20 μg/kg) and housing condition on the expression of LI in a CER paradigm. Lick suppression ratio values are presented for adult male (A) and female (B) rats when tested one week after the initial test. Values represent Mean ± SEM, asterisk indicates a significant difference between PE and NPE groups, illustrating the presence of LI ($p < 0.05$). n = 5-6 animals per group.

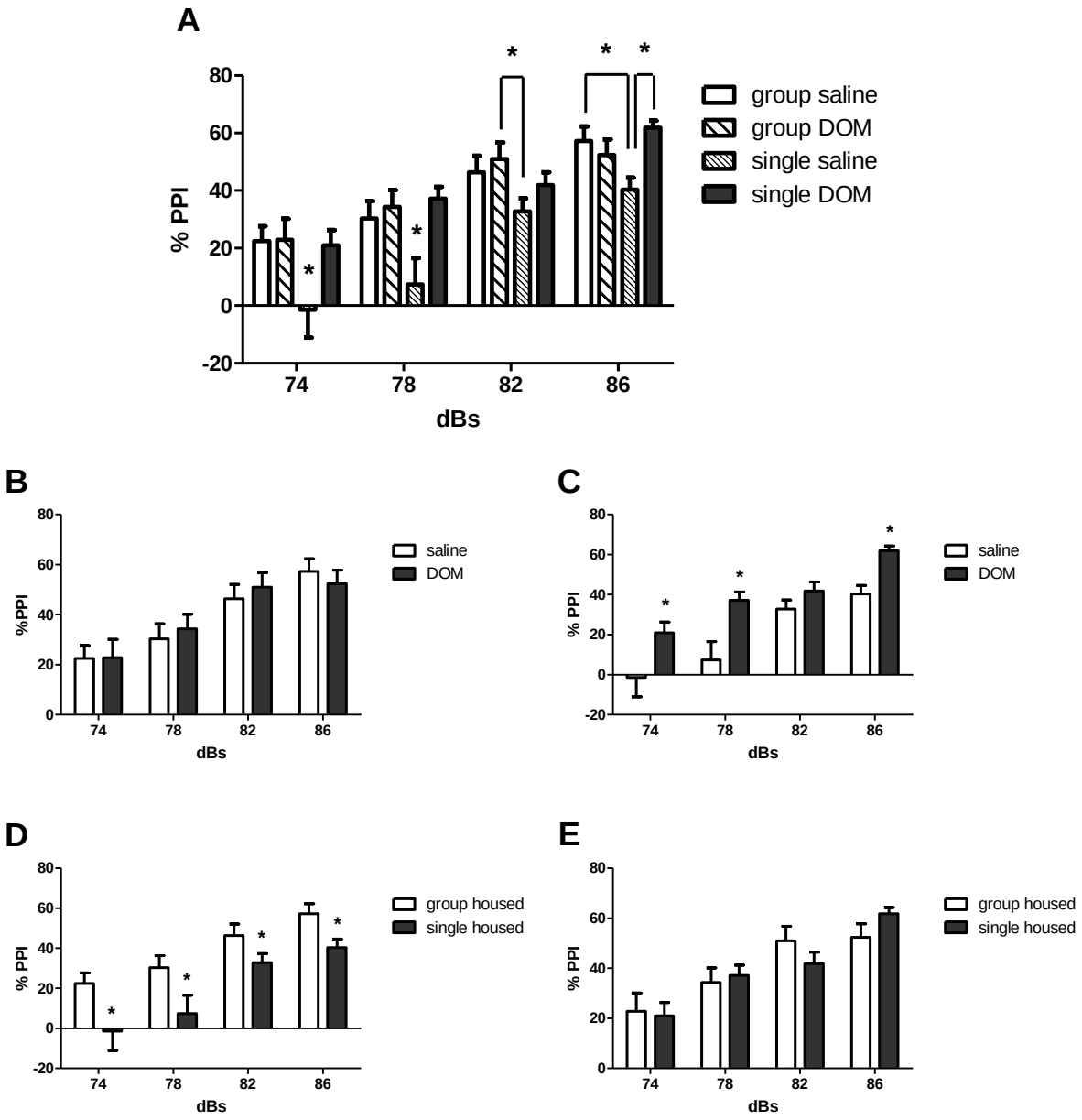

Figure 3.6 Mean ± SEM % PPI in male rats at various prepulse dB levels. Panel A displays data for all experimental group while panels B-E display the same data, broken down to illustrate the effect of drug treatment on group housed rats (B), the effect of drug treatment on isolation housed rats (C), the effect of housing condition on saline treated rats (D) and the effect of housing condition on DOM treated rats (E). Asterisk indicates a significant difference from the comparison group(s) ($p < 0.05$). n = 11-12 rats per group

(Figure 3.6B). Significant housing effects were found in the saline treated males (Figure 3.6D), with single housed rats showing significantly lower %PPI as compared to group housed rats at 74dBs (-1.380 ± 9.643 vs 22.472 ± 5.154) [t_{21} = -2.234, p = 0.018], at 78dBs (7.368 ± 9.138 vs 30.329 ± 6.052) [t_{21} = -2.129, p = 0.023], at 82dBs (32.791 ± 4.535 vs 46.338 ± 5.784) [t_{21} = -1.820, p = 0.042] and at 86dBs (40.385 ± 4.177 vs 57.296 ± 5.010) [t_{21} = -2.567, p = 0.009]. This housing effect was not observed in the DOM treated rats (Figure 3.6E). Analyses of other measures revealed no significant differences in initial startle, movement during testing, baseline start at the start of testing, baseline startle at the end of testing or startle habituation.

Aside from the expected dB effect [$F_{2.08,89.58}$ = 54.442, p < 0.001], no significant differences were observed in the female rats (Figure 3.7). No significant differences were observed in movement during testing, baseline startle at the end of testing or startle habituation. Females showed a significant drug treatment x housing effect for initial startle [$F_{1,43}$ = 4.180, p = 0.047] and a significant drug group effect for baseline startle at the beginning of testing [$F_{1,43}$ = 4.155, p = 0.048], but when these effects were investigated further using t-tests, no further significance was observed.

3.3.3.2 Analysis of Vmax

Analysis of the maximum startle amplitude of each animal during PPI testing (Vmax) revealed results very similar to the analysis of %PPI (section 3.3.3.1). Male rats showed a significant main effect for dB level [$F_{2.48,106.83}$ = 51.137, p < 0.001] and drug group [$F_{1,43}$ = 5.555, p = 0.023] as well as an interaction between drug group and housing [$F_{1,43}$ = 5.831, p = 0.020]. When these effects were further investigated using t-tests (Figure 3.8), significant drug effects were found in the single housed males, with

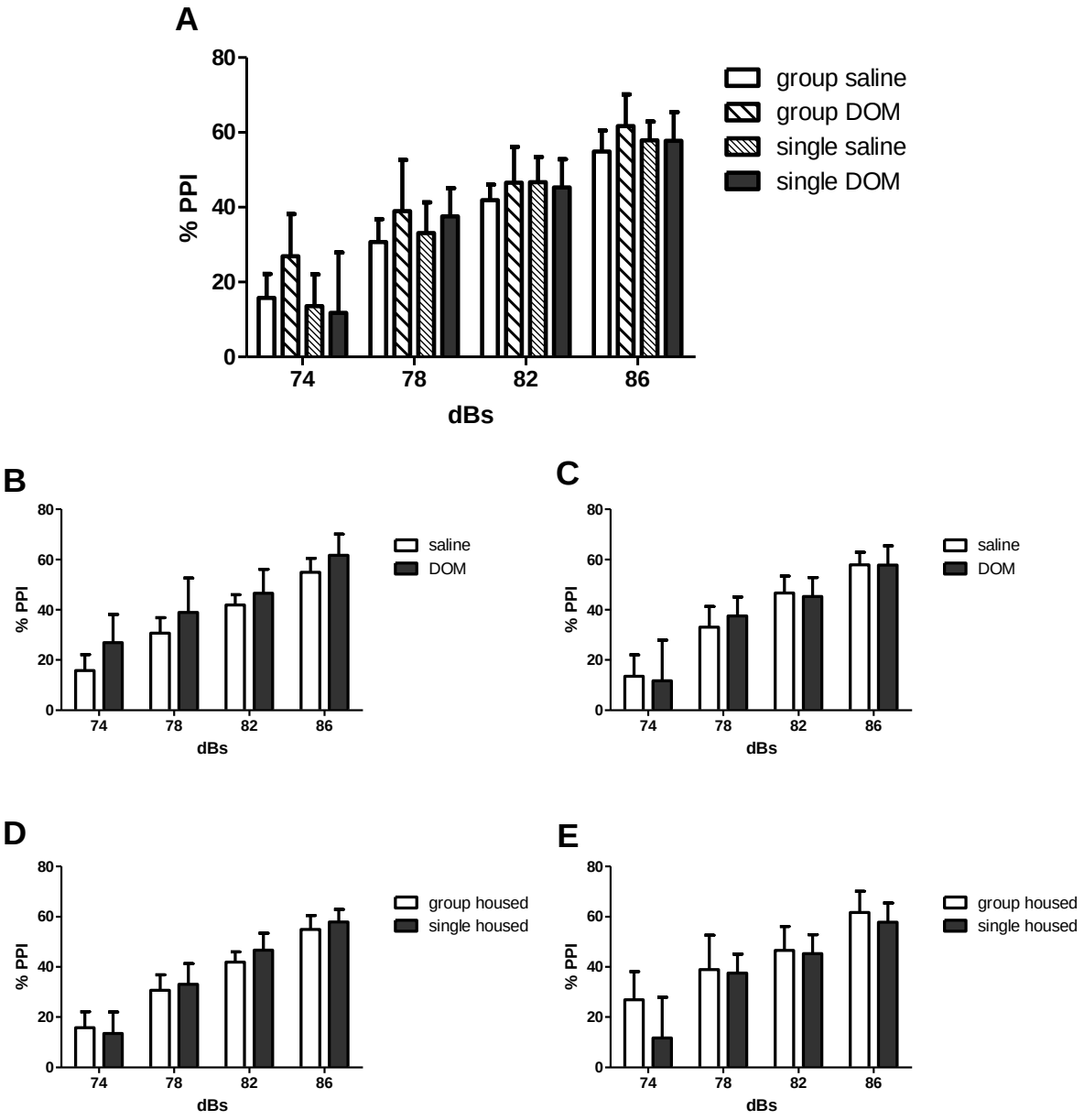

Figure 3.7 Mean ± SEM % PPI in female rats at various prepulse dB levels. Panel A displays data for all experimental group while panels B-E display the same data, broken down to illustrate the effect of drug treatment on group housed rats (B), the effect of drug treatment on isolation housed rats (C), the effect of housing condition on saline treated rats (D) and the effect of housing condition on DOM treated rats (E). Asterisk indicates a significant difference from the comparison group(s) ($p < 0.05$). n = 12 rats per group

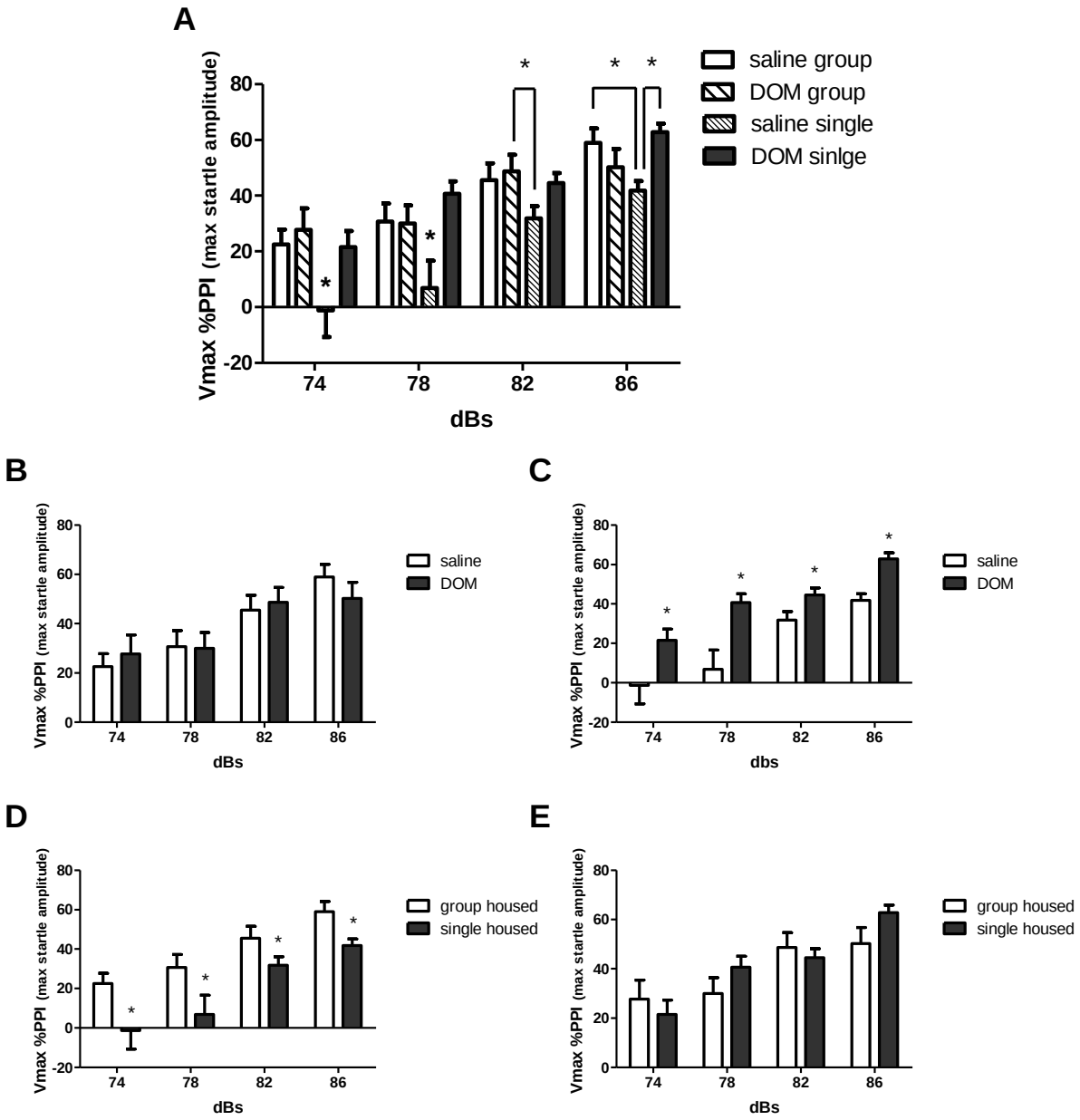

Figure 3.8 Mean ± SEM Vmax in male rats at various prepulse dB levels. Panel A displays data for all experimental group while panels B-E display the same data, broken down to illustrate the effect of drug treatment on group housed rats (B), the effect of drug treatment on isolation housed rats (C), the effect of housing condition on saline treated rats (D) and the effect of housing condition on DOM treated rats (E). Asterisk indicates a significant difference from the comparison group(s) ($p < 0.05$). n = 11-12 rats per group

SS males displaying significantly lowered Vmax (meaning they displayed less startle inhibition) compared to DS males (Figure 3.8C) at 74dBs (-1.194 ± 9.522 vs 21.520 ± 5.788) [t_{21} = 2.078, p = 0.025], at 78dBs (6.867 ± 9.748 vs 40.670 ± 4.449) [t_{21} = 3.247, p = 0.002], at 82dBs (31.866 ± 4.336 vs 44.556 ± 3.578) [t_{21} = 2.272, p = 0.017] and at 86dBs (41.812 ± 3.433 vs 62.778 ± 3.067) [t_{21} = 4.569, p < 0.001]. These drug effects were not seen in the group housed rats (Figure 3.8B). Significant housing effect were found in the saline treated males (Figure 3.8D), with single housed rats showing significantly lower Vmax than group housed rats at 74dBs (-1.194 ± 9.522 vs 22.535 ± 5.287) [t_{21} = -2.229, p = 0.019], at 78dBs (6.867 ± 9.748 vs 30.732 ± 6.497) [t_{21} = -2.070, p = 0.026], at 82dBs (31.866 ± 4.336 vs 45.531 ± 6.064) [t_{21} = -1.803, p = 0.043] and at 86dBs (41.812 ± 3.433 vs 58.933 ± 5.161) [$t_{18.83}$ = -2.762, p = 0.007]. This housing effect was not observed in the DOM treated rats (Figure 3.8E). Additional analyses revealed no significant differences for initial startle, baseline startle at the start of testing, baseline startle at the end of testing, habituation or movement during testing.

Aside from the expected significant difference for dB level [$F_{1.92,82.74}$ = 39.055, p < 0.001], no significant differences were observed in the Vmax of female rats (Figure 3.9). Further, no significant differences were observed in baseline startle at the end of testing, habituation or movement during testing. When assessing initial startle, a significant interaction between drug treatment and housing was found [$F_{1,43}$ = 5.036, p = 0.030] and a significant drug effect was found for baseline startle at the start of testing [$F_{1,43}$ = 4.278, p = 0.045] but when these effects were investigated further using t-tests, no significant differences between specific groups were observed.

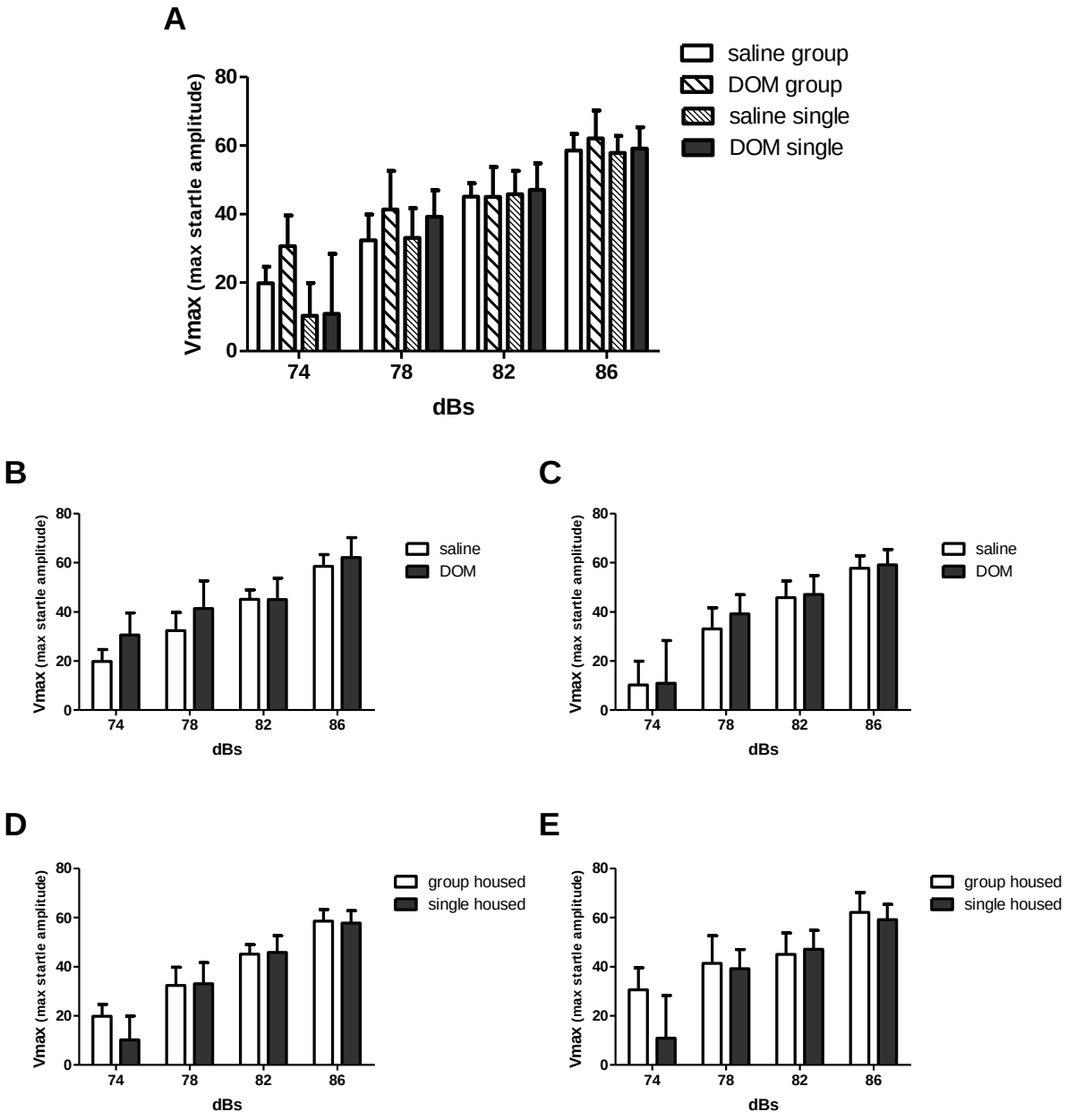

Figure 3.9 Mean ± SEM Vmax in female rats at various prepulse dB levels. Panel A displays data for all experimental group while panels B-E display the same data, broken down to illustrate the effect of drug treatment on group housed rats (B), the effect of drug treatment on isolation housed rats (C), the effect of housing condition on saline treated rats (D) and the effect of housing condition on DOM treated rats (E). Asterisk indicates a significant difference from the comparison group(s) ($p < 0.05$). n = 12 rats per group

3.3.3.3 Analysis of Tmax

Data were analyzed for the latency for animals to reach the point of maximum startle amplitude during PPI testing. Male rats showed a significant interaction effect for drug treatment by housing [$F_{1,43}$ = 4.996, p = 0.031]. When this effect was investigated further using t-tests (Figure 3.10), significant drug effects were found in the single housed males, with DOM treated rats startling fast than saline treated rats (Figure 3.10C) at 78dBs (-2.994 ± 1.501 vs 2.886 ± 1.633) [t_{21} = -2.656, p = 0.008] and at 86dBs (-1.850 ± 1.633 vs 3.007 ± 1.974) [t_{21} = -1.908, p = 0.035]. This effect was not observed in the group housed rats (Figure 3.10B). Significant effects of housing condition were also observed in the DOM treated rats with single housed males startling significantly faster than group housed males (Figure 3.10E) at 78dBs (-2.994 ± 1.501 vs 3.313 ± 2.018) [t_{22} = -2.508, p = 0.010] and 86dbs (-1.850 ± 1.633 vs 3.143 ± 1.700) [t_{22} = -2.118, p = 0.023]. These effects were not seen in the saline treated males (Figure 3.10D). No significant effects were found for initial startle, baseline startle at the end of testing, or movement during testing. A significant main effect of housing condition was found for the measures of baseline startle at the start of testing [$F_{1,43}$ = 8.149, p = 0.007] and habituation [$F_{1,43}$ = 4.518, p = 0.039] but when investigated further no significant group differences were found.

In female rats there were no significant main effects for any variable and no interactions between variables were present, although the variable for housing condition was approaching significance [$F_{1,43}$ = 3.993, p = 0.052]. In order to remain consistent with the analysis conducted for the males, these data were further investigated using t-tests (Figure 3.11). A significant effect for housing was found in the DOM treated females whereby the single housed rats (-2.562 ± 1.609) startled significantly faster than

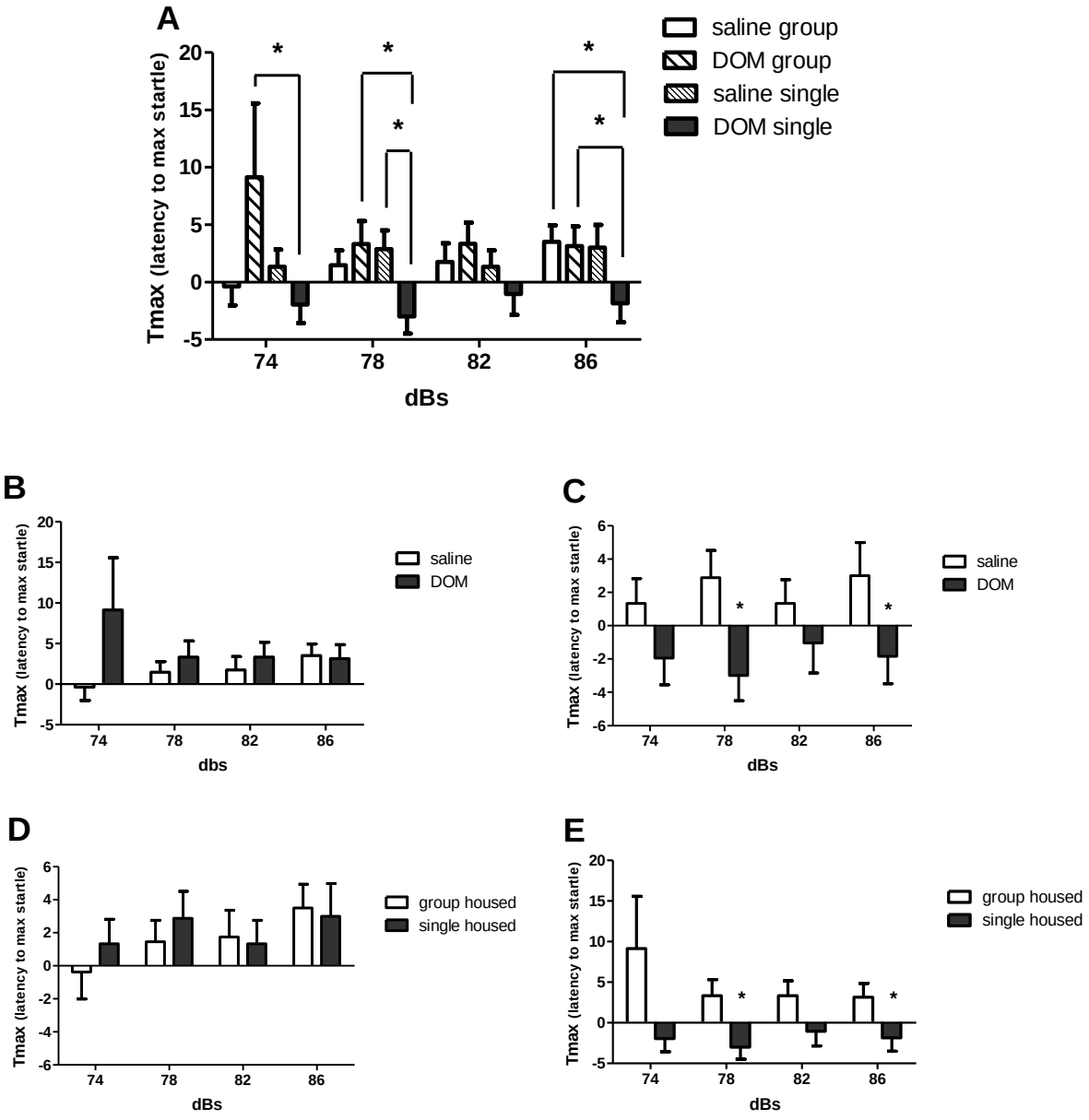

Figure 3.10 Mean ± SEM Tmax in male rats at various prepulse dB levels. Panel A displays data for all experimental group while panels B-E display the same data, broken down to illustrate the effect of drug treatment on group housed rats (B), the effect of drug treatment on isolation housed rats (C), the effect of housing condition on saline treated rats (D) and the effect of housing condition on DOM treated rats (E). Asterisk indicates a significant difference from the comparison group(s) ($p < 0.05$). n = 11-12 rats per group

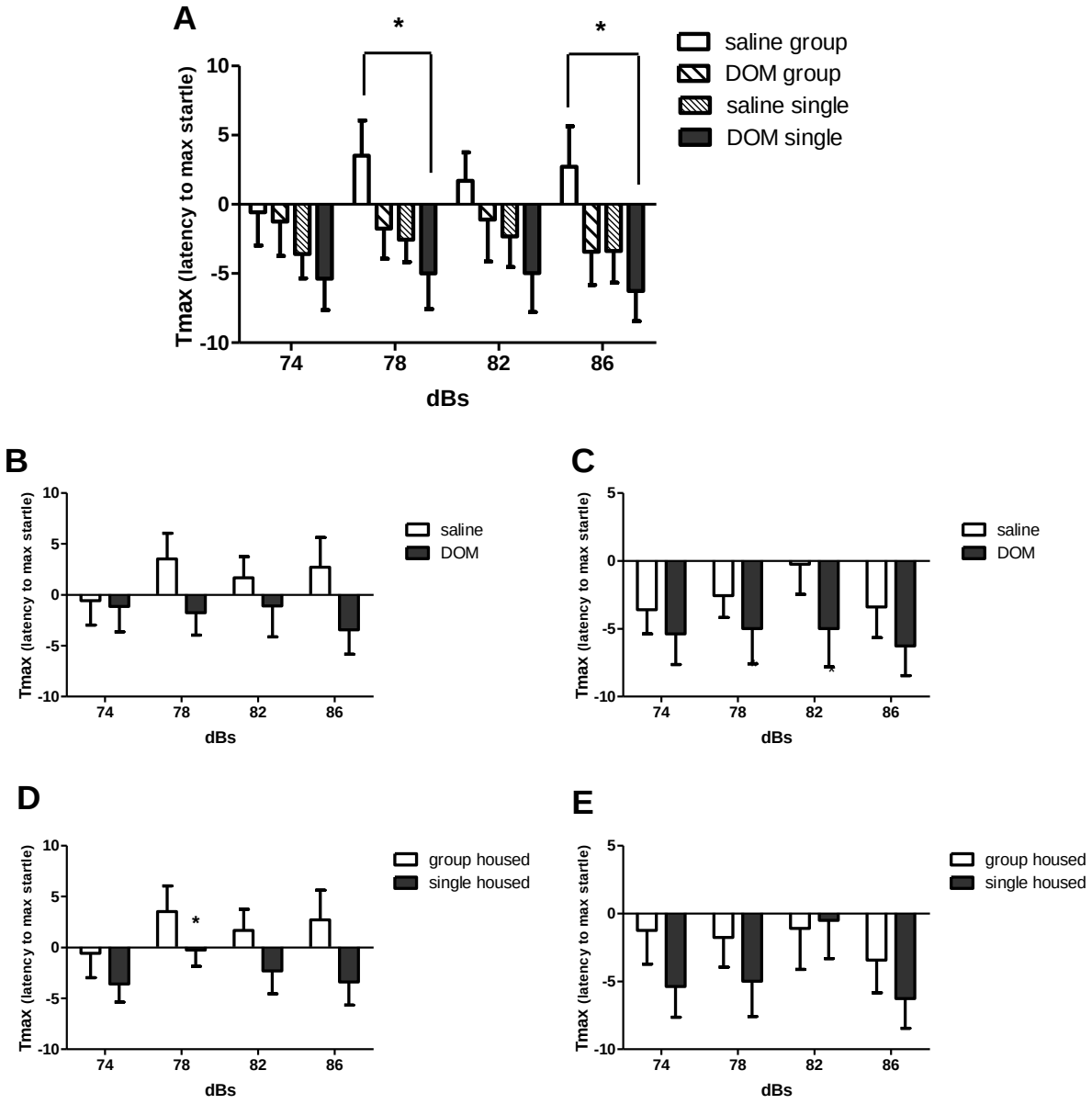

Figure 3.11 Mean ± SEM Tmax in female rats at various prepulse dB levels. Panel A displays data for all experimental group while panels B-E display the same data, broken down to illustrate the effect of drug treatment on group housed rats (B), the effect of drug treatment on isolation housed rats (C), the effect of housing condition on saline treated rats (D) and the effect of housing condition on DOM treated rats (E). Asterisk indicates a significant difference from the comparison group(s) ($p < 0.05$). n = 12 rats per group.

the group housed rats (3.532 ± 2.535) at 78dbs [t_{22} = -2.029, p = 0.028] (Figure 3.11D). No significant differences were found for initial startle, baseline startle at the start of testing, baseline startle at the end of testing, habituation or movement during testing.

3.4. Discussion

The aim of the present study was to investigate the effects of neonatal DOM treatment and isolation rearing on attentional processing as measured by LI and PPI. This study was conducted to learn more about how early life interventions might be useful either alone or together in modeling this particular aspect of schizophrenia in rats, as well as to provide a foundation for future mechanistic studies on the systems responsible for normal and abnormal attentional processing. The results show that sex, housing status and neonatal DOM treatment all affect LI and PPI and that the interactions between these variables are complex.

3.4.1 Latent inhibition

One of the more dramatic and consistent results obtained from this study was that neonatal treatment of male rats with DOM completely abolished the significant LI effect observed in saline treated rats at 48 hours regardless of housing condition (Figure 3.4A). Indeed, mean lick suppression ratios for DOM-treated males pre-exposed to the tone were almost identical to those obtained in non pre-exposed males treated with saline (Figure 3.4A). These data can be interpreted as evidence that at least in male rats, neonatal treatment with DOM is highly disruptive to the development of normal LI, and is consistent with data we have published previously on DOM-mediated changes in sensory gating (Adams *et al*, 2008a; Marriott *et al*, 2012) and other behavioural

correlates of schizophrenia (Adams *et al*, 2009; Burt *et al*, 2008a, 2008b; Doucette *et al*, 2007; Gill *et al*, 2012; Robbins *et al*, 2013; Ryan *et al*, 2011). While this abolition of LI in the DOM treated males could be due to any number of factors, it is possible that given the extensive involvement of DA and GABA in LI behaviour (see sections 1.3.4.1 and section 4.1), that one or both of those systems has been altered. This theory will be further explored in Chapter 4.

The effects of neonatal DOM on the development of LI in female rats at 48 hours produced a markedly different result. In females, both single and group-housed rats treated with DOM displayed significant LI (and both groups of saline-treated female rats showed a tendency toward significant LI) (Figure 3.4B). The reason for this sex-dependent effect of DOM on LI is unclear, but may be indicative of differential timing of key events in brain development between males and females. In the current study both male and female rats received DOM injections from PND 8-14. Therefore, if either the circuitry responsible for LI expression (see section 1.3.4.1), or the expression of proteins (e.g. receptors) responsive to DOM, differs temporally in female rats, the robust drug effect observed in males may be lost in females. Sex differences in LI behaviour have been observed in both the clinical population (Lubow *et al*, 2001), in healthy test subjects (Klosterhalfen *et al*, 2005), and in various animal models (Bethus *et al*, 2005). Moreover, there are recent data indicating that Glu receptor expression profiles differ temporally in male and female neonatal rats (Galanopoulou, 2008) as well as there being potential sex differences in the excitatory/inhibitory tone of the neonatal GABA system (Nuñez and McCarthy, 2007). Such sex differences in the major excitatory and inhibitory systems of the CNS could partially account for the effects observed but this requires further investigation. Additionally, part of the effect seen here is similar to what

we observed in a previous study, where male DOM treated rats showed decreased LI 24 hours following conditioning, but female DOM treated rats did not (Marriott *et al*, 2012). Furthermore, the presence of sex differences in adult rats treated neonatally with DOM following this same protocol has been well establish both behaviorally and histopathologically (Adams *et al*, 2008a, 2009; Burt *et al*, 2008a, 2008b; Doucette *et al*, 2003, 2007; Gill *et al*, 2010; Robbins *et al*, 2013; Ryan *et al*, 2011)

Social isolation has been reported previously to impair measures of attentional processing including LI, although results vary (Feldon *et al*, 1990; Han *et al*, 2012; Shao *et al*, 2009; Weiss *et al*, 2001; Wilkinson *et al*, 1994). In the current study, male rats that were group housed and received injections of saline exhibited a strong LI effect as displayed by a significant reduction in lick suppression following pre-exposure to the tone (Figure 3.4A). While the same general trend was observed in those animals that received saline but were housed in isolation (SS), there was no significant difference between the PE and NPE groups (Figure 3.4A). However, mean lick suppression ratios differed by more than two-fold in these two groups of SS male rats, suggesting that the failure to produce significant LI may be more a reflection of the increased variability in the number of licks in the NPE group. Single housing has been reported to produce changes in water consumption during testing paradigms without accompanying changes in water consumption in the home cage although results are contradictory with Jones *et al*. (1989) reporting that isolation reared rats showed decreased drinking whereas Hawken *et al*. (2013) reporting increased drinking. Regardless, altered water consumption might be impacting on the measures of LI in the male SS animals. Additionally, previously published data, using a taste aversion paradigm to asses LI, suggests that the housing effect may be a graded effect. In that study LI was produced in

male saline-treated animals reared in groups of two-three and deficits in LI were evident at 24 hours post exposure in their DOM treated counterparts (Marriott *et al*, 2012).

Data on housing effects obtained with female animals again provided very different results. When tested 48 hours post-conditioning, female rats that were either group or single housed and received injections of saline did not display statistically significant LI (Figure 3.4B) although it could be argued that the saline treated females (in both housing groups) are displaying a trend towards LI and that the lack a statistically significant LI effect in these groups may be a function of variability and small sample size. Indeed, data presented in Chapter 2 of this thesis confirmed previous unpublished findings in our laboratory demonstrating that LI is more difficult to produce in female rats and may require different paradigms than are needed with males.

It is uncommon for studies of LI to include more than one test session. However, other investigators have reported that the LI effect can persist for a very long time (Guanowsky *et al*, 1983), and given the theory that the lack of LI, as well as the abnormal persistence of LI might model different aspects of psychiatric disorders (Weiner and Arad, 2009; Weiner, 2003), a second test session conducted 7 days after the first was included in the current study. This was also consistent with our previous study that assessed the effect of neonatal DOM treatment on adult LI using a CTA paradigm, in which DOM treatment affected LI in male rats 24 hours post-conditioning (but not 1 week later), and affected LI in female rats 1 week post-conditioning (but not at 24 hours) (Marriott *et al*, 2012).

Therefore, to determine if the effects of DOM treatment and/or housing condition produced different effects after a one week delay, each rat was re-tested at 9 days post-conditioning (one week following the initial test). While the effects seen in

male rats at 48 hours post conditioning indicated that DOM treatment abolished LI (Figure 3.4A) results from the second test session suggested that there is an abnormal permanence of the LI effect as evidenced by a significant LI effect in the DS group (Figure 3.5A) while no LI effect is observed in any other group. While an explanation for this effect requires further study, one argument is that the effects of DOM manifest mainly in the short-term whereas social isolation produces longer lasting disruptions of LI, and that the effect of social isolation on LI at 48 hours was simply masked by the profound disruption of LI induced by neonatal DOM (i.e. a "plateau effect"). If this is the case, it lends further support to the idea that various aspects of LI might illustrate various aspects of schizophrenia, and indicates the further potential of neonatal DOM treatment to comprehensively model this disorder. When females were tested again one week later, no significant LI effects were seen in any group (Figure 3.5B).

3.4.2 Prepulse inhibition

Both neonatal DOM treatment and social isolation rearing had a profound effect on the PPI of male and to a lesser extent, female rats, but these effects were different for the various measures of PPI.

In saline treated male rats, social isolation resulted in significantly lowered %PPI at the 74, 78 and 86 dB prepulse levels (Figure 3.6). Similarly, social isolation resulted in significantly lowered maximum startle (Vmax) at the 74, 78 and 86 dB level in saline treated males (Figure 3.8). This effect is consistent with what has been previously reported (Domeney and Feldon, 1998; Geyer *et al*, 1993; Stevens *et al*, 1997) and confirms that our social isolation paradigm is producing strong and consistent deficits in measures of the amplitude of PPI.

Interestingly, this expected PPI deficit due to social isolation was not observed in the DOM treated rats. In fact, there was no significant difference in the %PPI or Vmax behaviour between the group housed and social isolation housed DOM rats, suggesting that neonatal DOM treatment may result in animals being refractory to the effect of isolation rearing on PPI. One potential reason for this finding could be that DOM treated rats have a different response to social isolation compared to saline treated rats. Our laboratory has previously observed that neonatal DOM treatment results in altered social interaction (McInnis, 2009; Ryan *et al*, 2011) and has reported an altered response to novelty (Burt *et al*, 2008a). Because the effects of social isolation or social housing rely on both the behaviour of the test subject as well as the other animals, if the DOM treated rats experience social isolation and social activity in a different way than control animals, it could theoretically result in housing condition having a different effect on them. For example, if DOM treated rats are generally less interested in social interaction, isolation rearing may not be as stressful for them as it would for saline treated rats.

Another interesting result of this study is that we did not observe the reduction in %PPI due to DOM treatment that we have previously observed and reported. In our previous studies we have reported that neonatal DOM treatment produces a decrease in %PPI in male rats (Adams *et al*, 2008a) when tested during the light phase of the light/dark cycle, and in female rats (Marriott *et al*, 2012) when tested during the dark phase of the light/dark cycle. Such differences in sex and time of day testing on PPI are not surprising in that many studies have found sex differences in PPI in both humans (Kumari *et al*, 2004) and rats (Lehmann *et al*, 1999), with males consistently displaying greater PPI, particularly at lower prepulse levels. The time of day during which testing occurs has also been found to affect PPI in experimental animals, with a study in our lab

on female PPI behaviour finding that although startle and PPI were not affected by the estrous cycle, the time of day during which testing occurred could have an effect, with greater %PPI demonstrated when the rats were tested during the dark cycle (Adams *et al*, 2008b). One possible reason for the lack of a PPI deficit observed in the group housed animals in the current study may be the age at testing. In our previous studies PPI was tested at PND 90 whereas in the present study the animals were approximately 135 days old by the time PPI testing began. Thus a DOM-induced PPI deficit may present only in early adulthood and disappear as the animal ages.

In the clinical population of people with schizophrenia, PPI deficits are reliably observed in non-medicated, first-episode people (Ludewig *et al*, 2003). While it has been suggested that PPI deficits are present at the early stages of diagnosis but may change as the disease progresses, some studies have indicated that this is not the case by observing similar PPI behaviours in people experiencing acute, first-episode psychosis and those that are clinically stable (Parwani *et al*, 2000). However the results of such studies are confounded by the fact that most long-term patients are receiving treatment, making it difficult to dissociate age-related changes from treatment-dependent changes.

Another possibility is that the housing conditions differed between the current and previous studies. In our previous studies, animals were housed in groups of 2 or 3 rats per cage while in the present study, animals in the isolation group were housed alone and animals in the social group were housed in groups of 4. As the results of this study illustrate, housing conditions can have a dramatic effect on PPI behaviour. Studies that aim to investigate the effect of social vs. isolation rearing on PPI usually house animals in the social condition in groups of 3-4 (Bakshi *et al*, 1998; Domeney and Feldon, 1998; Varty and Geyer, 1998; Varty *et al*, 1999; Weiss *et al*, 2000) as was done

in the current study. Perhaps there is some lower limit to the number of animals needed in a cage in order to produce a true "social housing" environment and the housing conditions in our previous studies, where many animals were housed primarily in groups of 2, has contributed to an altered PPI behaviour.

An interesting study by Weiss *et al*. (1999) found that the PPI deficits caused by social isolation housing occurred in rats raised in solid bottomed cages, but not rats raised in grid-floor cages. It is possible that raising animals in grid-floor cages represented a form of chronic mild stress that made the rats refractory to the normal isolation induced PPI deficits. Furthermore, this finding lends support to the idea that social isolation rearing induced deficits in PPI are fragile and may offer an explanation as to why we observed PPI disruptions in those isolation housed animals that were treated with saline, but not those that were treated with DOM.

The analysis of Tmax (the latency to the point of maximum startle amplitude) provided very different results from the first two measures. Both social isolation and DOM treatment appeared to decrease the latency to startle in an additive manner in both male and females rats. As illustrated in Figure 3.10A, male rats who were treated with DOM and who were housed in isolation after weaning displayed significantly faster startle times on PPI trials versus those animals in other groups, whose startle times were very similar for most dB prepulse levels. Similar results were seen in the females (Figure 3.11A) where once again, neonatal DOM treatment and isolation rearing appear to be having an additive effect and produced faster startle times on prepulse trials. This effect was significant at the 78 and 86 dB levels, although the other dB levels displayed a similar trend. Furthermore, as there were no significant differences in latency to startle when measuring the baseline startle behaviour (without a prepulse) these effects cannot

be attributed to baseline differences in startle behvaiour in the case of either sex. While the meaning of these reduced latency values is unknown, it suggests alterations to the inhibitory processes regulating startle latency.

Analysis of the latency to maximum startle is not reported in most of the published literature. Lyall *et al.* (2009) found that rats treated neonatally with the NMDA receptor antagonist MK801 and tested at PND56, displayed a prepulse-induced delay in startle response time, but found no changes in average startle amplitude or peak startle amplitude. In a study on the effects of iron deficiency conducted by Burhans *et al.* (2006), both male and female rats that were fed an iron deficient diet from weaning until adulthood displayed lowered baseline startle amplitudes, but showed no changes in maximum startle amplitude on PPI trials. Furthermore, Tmax was significantly longer on PPI trials in iron deficient female rats, but the effect was not observed in iron deficient male rats (Burhans *et al*, 2006). Other studies have measured Tmax, but only for measures of baseline startle, not for PPI. For example, a study conducted by Clark *et al.* (2005) showed that C57BL/6J mice display a significant lowering of the baseline startle reflex and an increased latency to peak startle amplitude during baseline startle tests in response to the cholinesterase inhibitor physostigmine. Additionally they found significantly increased PPI following treatment with physostigmine, although it is unclear if this result was obtained by measuring the average startle amplitude or the peak startle amplitude, and they did not report on startle latency for the PPI trials. There are also a small number of studies that did test for Tmax according to the written methods, but did not discuss the results in publication. While including the latency to maximum startle is uncommon in the literature, the findings of this study highlight the future

potential of using this measure to more fully understand the outcome of experimental manipulations that may affect PPI.

3.4.3 Conclusion

Based on the results described we can conclude that neonatal DOM treatment abolishes LI behaviour in adult male rats, regardless of housing when tested 48 hours after conditioning. This effect is not observed in female rats treated with an identical paradigm, further reinforcing the need to study both male and female animals in subsequent studies. Social isolation rearing from weaning until adulthood may also have an effect on LI, but does not alone appear to produce the dramatic reduction in LI observed in other models, although our data do support further study on whether social isolation produces abnormally persistent reductions in LI, as well as whether the effects of neonatal DOM and social isolation are synergistic either through the same or different pathways mediating this measure of attentional processing. Neonatal DOM treatment has a strong effect on all measures of PPI assessed in this study although it appears to be countering the ability of isolation rearing to affect startle amplitude and working in an additive manner with isolation rearing to affect startle latency.

Furthermore, the results of this study lend support to the practice of conducting multiple assessments of LI behaviour after conditioning has occurred. Testing LI once only a short time following conditioning may result in missing important information about how these behaviours are expressed over time. Additionally the results of this study support the addition of an analysis of latency to maximum startle when testing PPI. Finally, the ability of neonatal DOM treatment to produce persistent changes when combined with another model is encouraging and illustrates the potential of combining

this model with other models in pursuit of a 2-hit (or multi-hit) model of neuropsychiatric disorders.

3.5 References

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Chapter 4

Effects of chemically and environmentally induced changes in brain development on neurochemical measures of attentional processing

The data presented in this chapter is being included in a manuscript to be submitted to Behavioural Brain Research in May of 2014.

Summary

Neonatal DOM treatment and social isolation rearing both lead to significant and long lasting changes in behavioural measures of attentional processing in adult rats. To continue investigating these effects, neurochemical analyses of putative neurotransmitter systems in relevant brain areas were conducted on tissue obtained at the conclusion of behavioural testing. Protein markers of interest were selected based on the current understanding of the brain areas and systems involved in the observed behavioural changes, their involvement in disorders observed in the human clinical population, and the results of previous findings in these models. From PND 8-14, male and female Sprague-Dawley rats were treated with either 20µg/kg of the Glu agonist DOM or with saline (controls). Upon weaning at PND 21 animals were housed in either a socially isolated condition (1 animal per cage) or a social housing condition (4 animals per cage). Following behavioural testing in adulthood (described in Chapter 3), brain tissue was obtained and assayed semi-quantitatively for protein markers of both DA and GABA system activity using Western blot analysis. Results indicated some interesting trends but no significant differences were found in the overall expression of D1 receptor, D2 receptor, tyrosine hydroxylase, GAD65 or GAD67 protein in either the PFC or hippocampus of any experimental group. These results indicate that other neurotransmitter systems and/or brain areas are likely involved in the behavioural changes that were previously observed. It is also possible that other methods or increased statistical power may be required to detect these changes.

4.1 Introduction

The discovery that both neonatal treatment with low-dose DOM and social isolation rearing produce significant and long lasting behavioural changes raises the obvious question of what specifically has been altered in the brains of these animals to cause the observed behavioural effects? While it is possible that the early life interventions described in Chapter 3 might have affected the brain in a multitude of ways, an inference of which systems and areas might be affected can be made by considering both the previous literature on these models as well as our understanding of how the brain is involved in the observed behavioural changes.

The extensive literature documenting the involvement of the DA system in many facets of abnormal LI behaviour makes it an obvious target for investigating the basis of the LI abnormalities observed in Chapter 3. Furthermore, while the administration of DA agonists leads to disrupted LI (as seen in male DOM-treated rats when tested 48 hours post-conditioning, see Figure 3.4A), the administration of DA antagonists results in the abnormal permanence of LI (seen in the DOM treated females at 48 hours and the DOM treated/ single housed males at 9 days post-conditioning, see Figures 3.4B and 3.5A) (Weiner and Arad, 2009). Consequently, whether increased or decreased, changes in dopaminergic system markers were considered likely candidates for investigating the neurobiological basis of the observed behavioural changes.

In addition to DA, the GABAergic system has been previously implicated in PPI behaviour (Bast and Feldon, 2003; Guo *et al*, 2013; Heldt *et al*, 2004).While the specific role(s) of GABA in modulating PPI are not known, it is reasonable to assume that the transmitter functions to inhibit neurotransmission. In Chapter 3, DOM-treated/single housed animals showed a decreased latency to maximum startle in PPI (see Figures 3.10

and 3.11). Disinhibition due to a reduction in GABA system function could be one explanation for this result.

4.1.1 Dopamine

Historically, DA was the first neurotransmitter system to be implicated in schizophrenia, with the predominant hypothesis being that symptoms of the disorder were caused by excessive DA transmission in the forebrain (Snyder, 1976). It is, therefore, not surprising that much of the early research on this disease concentrated on the DA system. Indeed, when Solomon *et al.* (1981) first proposed the LI model of schizophrenia they showed that proper functioning of the DA system is required for normal LI behaviour. Weiner *et al.* (1981, 1984, 1988) also conducted a series of studies demonstrating how the administration of the DA agonist amphetamine, which produces and exacerbates some of the positive symptoms of schizophrenia (Angrist *et al*, 1974, 1980; Janowsky and Davis, 1976; Lieberman *et al*, 1987; Snyder, 1973), resulted in a lack of LI. Additionally, it has been shown that potentiation of LI behaviour can be produced by the administration of the D2 receptor antagonist haloperidol (Christison *et al*, 1988; Weiner and Feldon, 1987; Weiner *et al*, 1987). These animal studies implicating the role of the DA system in LI are further supported by similar effects being observed in the human clinical population (see Weiner 2003 for review).

Dysfunction of the DA system has also been implicated in altered PPI behaviour. A study by Swerdlow *et al.* (1986) was the first to suggest a role for the DA system in PPI by showing that inducing hyperactivity of DA in the forebrain produced PPI deficits similar to those seen in the clinical population. In a study which further characterized the role of DA in PPI, Mansbaeh *et al.* (1988) found that treatment with the DA agonists

apomorphine and d-amphetamine resulted in a significant decrease in PPI, while haloperidol prevented the apomorphine-induced PPI deficit but had no effect when administered alone. Furthermore, the DA system has been extensively implicated in attentional processing, particularly with regard to the behaviours discussed in this chapter (see section 1.3.4.1 for more on LI and 1.3.4.2 for more on PPI).

Previous work with the neonatal DOM model used herein has also produced data that imply alterations to the DA system. A study by Robbins *et al.* (2013) found that neonatal DOM treatment lead to significantly less tyrosine hydroxylase (TH) immunoreactivity in the right mPFC of male rats, and significantly greater TH immunoreactivity in the left core and right shell of the NAc in female rats. Although Gill *et al.* (2012) found no statistically significant differences in protein markers of DA function, it was noted that neonatal DOM treatment resulted in a consistent trend of lowered D2 receptor protein (assessed by Western blot) of the cytosol/membrane fraction of the anterior portion of the brain (which included the PFC), in male DOM treated rats. Animals in both of these studies received the same DOM treatment protocol described herein, but rats were housed in smaller groups of 2-3 animals.

Changes in the DA system have been also been observed in rats who were reared in social isolation but results have been varied and sometimes contradictory. An in-vivo microdialysis experiment conducted by Jones *et al.* (1992) found that rats reared in isolation showed higher DA levels in both the dorsal striatum and NAc following systemic administration of 2 mg/kg d-amphetamine, as well as higher post-mortem DA concentrations in the mPFC, but not in subcortical areas. In contrast, a study by Leng *et al.* (2004) found no change in DA, DOPAC or homovanillic acid in the mPFC, NAc or caudate putamen of rats. One possibility is that these DA changes are strain dependant,

as a study by Trabace *et al.* (2012) found increased DA and a reduction in DA turnover in a variety of brain areas including the PFC, NAc, hippocampus and striatum, but these results were observed only in Lister Hooded rats and not in Wistar rats.

4.1.2 GABA

Another neurotransmitter system that has been implicated more recently in schizophrenia is the GABA system (Benes and Berretta, 2001). GABA is the major inhibitory neurotransmitter in the mammalian CNS, with GABAergic interneurons exerting inhibitory control over circuits throughout the brain. Alterations to a variety of GABA system markers have been found in the post-mortem brains of people with schizophrenia, particularly in the hippocampus. These changes include decreased somatostatin and parvalbumin containing interneurons, as well as decreases in glutamic acid decarboxylase (GAD) (Konradi *et al*, 2011). In the mammalian CNS, GAD is the enzyme responsible for converting glutamate into GABA and occurs in two isoforms, GAD65 and GAD67 (Soghomonian and Martin, 1998).

While the role of GABA in PPI has not been investigated as extensively as the role of DA, studies have suggested that this system plays an important role in PPI behaviour, particularly within the hippocampus and the NAc (Bast and Feldon, 2003; Swerdlow and Geyer, 1998) (see section 1.3.4.2 for more on the role of GABA in attentional processing in PPI). Specifically, Guo *et al.* (2013) showed that inducing a temporary decrease in hippocampal neurogenesis in adolescent mice lead to a PPI deficit in adulthood, as well a reduction in PPI-related activation of hippocampal and prefrontal neurons. This effect was reversed by bilateral infusion of a $GABA_A$ receptor agonist into the hippocampus. They also found a reduction of GABAergic inhibitory neurons in the

dentate gyrus. Additionally, both the PPI deficits and the decrease in GABAergic neurons were avoided when mice were exposed to environmental enrichment during adolescence (Guo *et al*, 2013). A study that assessed the effect of genetic GAD65 knockout on mice found PPI deficits that were reversed by the atypical antipsychotic clozapine (Heldt *et al*, 2004).

Assessment of the role of GABA in regulating LI behaviour has produced conflicting results. A study by Delamater *et al.* (2009) reported that LI behaviour is not mediated by GABAergic mechanisms because systemic injections of a $GABA_A$ receptor inverse agonist failed to affect LI behaviour. Another study showed that a reduction in GABA activity in the PFC did not affect LI (Enomoto *et al*, 2011). However, Bitanihirwe *et al.* (2010) reported that prenatal immune activation in mice lead to both LI changes (abnormally enhanced LI) and a reduction of GABAergic markers in the hippocampus, but not the PFC. See section 1.3.3.3 for more on the role of GABA in the theoretical framework of attentional processing.

Previous results at UPEI have indicated that neonatal DOM treatment may have a long lasting effect on the GABA system. In a study that investigated a number of GABA system markers in the hippocampus of rats treated neonatally with DOM and housed in groups of 2-3 animals per cage, Gill *et al*. (2010) found decreased GAD65/67 combined immunostaining in the ventral dentate gyrus (females) and ventral CA3 area (males) of the hippocampus. They also found that DOM treated rats displayed a significantly lower number of parvalbumin containing neurons in the dorsal dentate gyrus, the mid dentate gyrus and the mid CA3 subfield. These results were often present in only one sex. Additionally, they found no change in the number of somatostatin containing neurons in any brain area (Gill *et al*, 2010). A small study by (Adams-

Marriott, 2009) which used 120 day old rats that were housed in groups of 2-3, found a significant decrease in GAD67 immunostaining in the CA3 of the left hemisphere of female adult rats treated neonatally with DOM.

The effect of social isolation rearing on GABAergic system function has been minimally investigated with a study by Harte *et al*. (2007) indicating that rats reared in isolation exhibited a reduction of parvalbumin and calbindin immunoreactive cells in the hippocampus, as well as PPI deficits.

The purpose of the experiments in the current chapter was to assess a variety of protein markers for both DA and GABA systems in the brains of adult animals that had been treated neonatally with DOM or saline, and housed either in isolation or in groups following weaning. Protein markers of interest were selected based on the current understanding of the brain areas and systems involved in the observed behavioural changes (Chapter 3), their involvement in disorders observed in the human clinical population, and the results of previous findings in these models (both social isolation and neonatal DOM treatment).

4.2 Materials and methods

4.2.1 Experimental animals and tissue processing

Experimental animals were the same rats used for behavioural testing in Chapter 3. When testing was completed animals were euthanized and tissue was obtained for analysis of proteins of the systems of interest. Brain tissue from 45 animals was processed for protein analysis through Western Blot with both sexes and all treatment groups being equally represented giving an n = 4 for each group in each sex. Animals were deeply anesthetized with isofluorane and immediately decapitated. Each brain was

rapidly extracted from the skull and the hemispheres were separated. The PFC and hippocampus were dissected out and flash frozen by immersion in liquid nitrogen. Tissue was then stored at -80^{o}C until needed. All procedures were conducted experimenter blind, according to the guidelines established by the Canadian Council on Animal Care and were approved by the Animal Care Committee at the University of Prince Edward Island.

4.2.2 Protein isolation and assay

Each sample was weighed and placed in 20ml/g of ice cold homogenization buffer (HB) (pH 7.4; 10mM TrisHCL, 300mM sucrose, 2mM EDTA) with Roche protease inhibitor (30µl/ml HB) (Roche Applied Science, IN, USA). Tissue was homogenized using a Polytron 3100 tissue tearer (Brinkman Instruments, ON, Canada) at 30,000 rpm for 30 seconds. The homogenate was then centrifuged at 2300 rpm for 20 minutes at 4^{o}C. The resulting supernatant, which contained both the membrane and cytosol fractions, was divided into 100 µl aliquots and stored at -80^{o}C for later analysis. All samples were assayed using the Bradford method to assess the concentration of total protein present.

4.2.3 Western blotting procedure for dopaminergic markers

Samples were mixed with Laemmli buffer with beta-mercaptoethanol (0.05%) added, and denatured by boiling for 3 minutes. Forty µg of each sample was loaded on to 8% bis-acrylamide gels and protein separated through electrophoresis (Bio-Rad PowerPac HC High-Current Power Supply, Bio-Rad Laboratories Inc., ON, Canada) at 0.06 constant amperage and a maximum of 200 volts. A standard sample common to all

gels was loaded into the first lane of each gel in order to control for inter-gel variations. Protein was then transferred on to PVDF membranes using a 0.2 constant amperage, a maximum of 25 volts and a maximum of 50 watts for 30 minutes (Bio-Rad PowerPac HC High-Current Power Supply, Bio-Rad, ON, Canada). All blots were notched for identification purposes.

Following blocking with 5% milk in PBS-T for 1.5 hours at room temperature, blots were incubated overnight in rabbit anti-D2 (1:500, Alomone Labs, Jerusalem, Israel), rabbit anti-D1 (1:1000, Alomone Labs, Jerusalem, Israel) mouse anti-tyrosine hydroxylase (1:1000 or 1:500, Millipore, MA, USA) or mouse anti-β-actin (1:5000, Sigma-Aldrich, USA) at 4^{o}C. The following day, blots were rinsed 3 times each in 10ml of PBS-T for 10 minutes. Blots were then incubated with the appropriate secondary antibody (rabbit 1:5000, rabbit 1:5000, mouse 1:5000 and mouse 1:20,000 respectively, Sigma-Aldrich, USA) for 1.5 hours at room temperature. An additional 3 PBS-T rinses were then conducted and protein bands were visualized by a one minute incubation in a chemiluminescent detection reagent (ECL Prime, Amersham, GE Healthcare Life Sciences, PQ, Canada) followed by UV exposure using the UVP BioSpectrum Imaging System (UVP, CA, USA). Optical density of the bands was measured using ImageJ (NIH) with values being normalized against β-actin and the control sample common to all blots.

4.2.4 Western blotting for GABAergic markers

4.2.4.1 Optimization of protein loading for quantification

Subsequent to the analysis of tissue samples for dopaminergic markers (see section 4.2.3) a report by Taylor *et al.* (2013) described how the assessment of optimal

protein loading for quantification purposes as an essential step in Western blotting, with excessive protein loading into a gel producing inaccurate quantification results. This is of particular concern with loading controls such as β-actin that are generally highly expressed. In such cases, consistent band densities among the sample lanes may not be the result of consistent protein expression among samples but rather may be caused by overloading of the gel, producing protein densities that are outside the linear dynamic range for accurate detection.

In order to avoid this issue and to determine the quantitative linear dynamic range, a representative sample was created by pooling a portion of all prepared samples for a given brain area. From this representative sample, a 2-fold dilution series over 12 dilutions was created (samples were diluted with HB), beginning at 80µg. A separate standard curve of band density vs. protein load was created for each primary antibody to be used.

The optimal protein load for GAD65 and GAD67 was determined to be approximately 20µg (see Figure 4.1 for representative figures). At this concentration, sample bands were found to be detectable, but not saturated. However, depending on the antibody concentrations used, loading this amount of protein may produce band saturation for β-actin. Therefore, various primary and secondary antibody concentrations were tested with β-actin (see Figure 4.2) and antibody concentrations chosen that would not result in oversaturation.

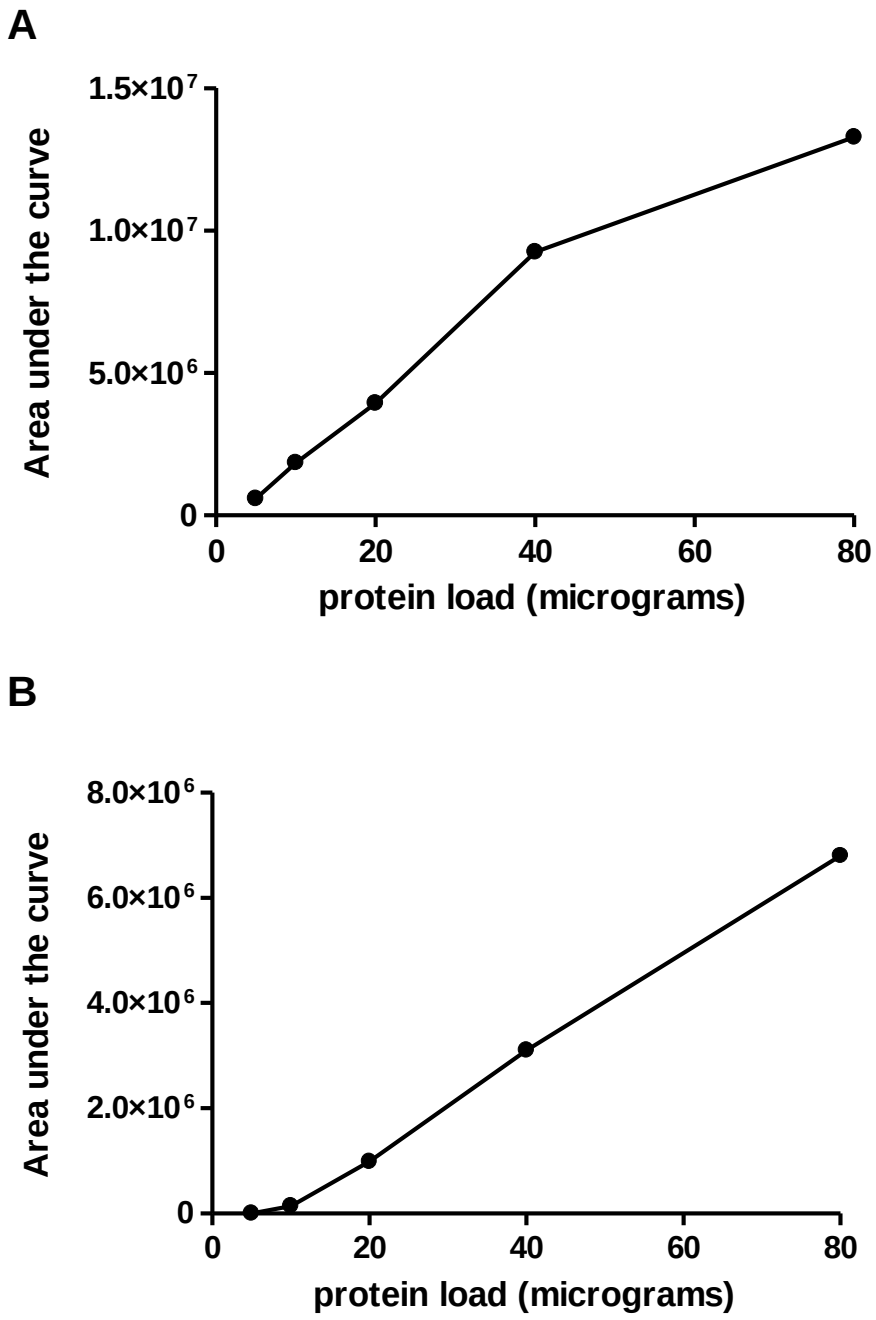

Figure 4.1 Quantification of serial 2-fold dilutions to define the linear dynamic range for Western blot detection for GAD65 protein (A) and GAD67 protein (B).

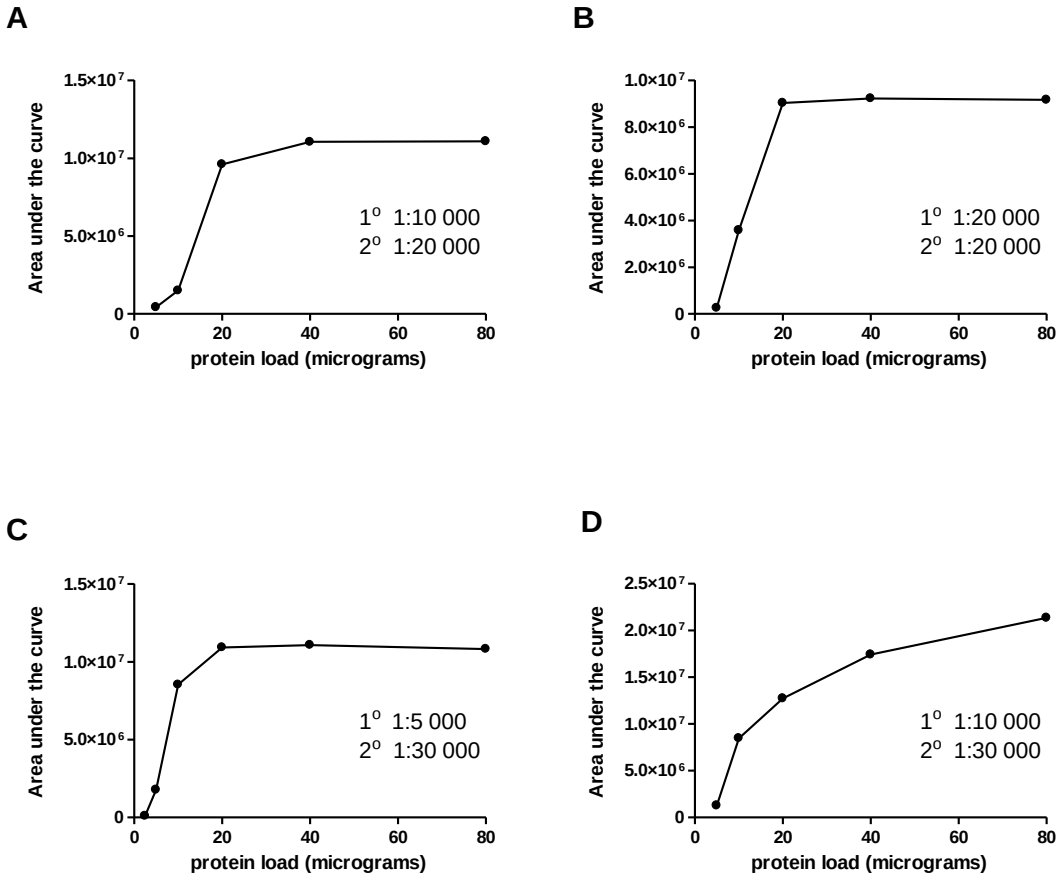

Figure 4.2 Various primary and secondary antibody concentrations to define the linear dynamic range of Western blot detection for β-actin protein.

4.2.4.2 Procedure

Samples were mixed with Laemmli buffer (Bio-Rad, Ontario, Canada) with beta-mercaptoethanol (0.05%) added, and denatured by boiling for 3 minutes. Twenty µg of each sample was loaded into 10% bis-acrylamide Bio-Rad Mini-PROTEAN TGX stain free gels (Bio-Rad, ON, Canada) and run at a constant of 200volts. A standard sample common to all gels was loaded into the first lane of each gel to control for inter-gel variations. Protein was then transferred onto PVDF membranes using a Bio-Rad TransferBlot Turbo Transfer System (Bio-Rad, ON, Canada). All blots were notched for identification purposes.

Following blocking with 5% milk in PBS-T for 1.5 hours at room temperature, blots were incubated overnight in mouse anti-GAD67 (1:2000, Millipore, USA) or rabbit anti-GAD65 (1:1000, Abcam, ON Canada), at 4^{o}C. The following day, blots were rinsed 3 times each in 10ml of PBS-T for 10 minutes each rinse. Blots were then incubated in the appropriate secondary (mouse 1:5000 or rabbit 1:5000, Sigma-Aldrich, USA) for 1.5 hours at room temperature. Another 3 PBS-T rinses were then performed and protein band detection was conducted by a 1 minute incubation in a chemiluminescent detection reagent (Bio-Rad Clarity ECL substrate, Bio-Rad Laboratories, ON Canada) followed by UV exposure using a Bio-Rad ChemiDoc MP Image System (Bio-Rad Laboratories, ON Canada). Optical density of the bands was measured using ImageLab (version 4.1, Bio-Rad Laboratories, Bio-Rad, ON Canada) with values being normalized against β-actin and the control sample common on all blots. See Appendix B for more information on quantification.

4.2.5 Data analysis

Unless otherwise stated, 2-way ANOVAs were used (drug treatment X housing condition), with repeated measures (hemisphere) where appropriate (SPSS Version 19). Post-hoc comparisons were conducted using Bonferroni t-tests, with Levene's test for equality of variance used where appropriate. A result of $p < 0.05$ indicated significance. Data for male and female animals were analyzed separately in order to remain consistent with the behavioural analyses, and because sex differences in protein expression in response to neonatal DOM treatment have been previously been observed (Adams-Marriott, 2009; Gill *et al*, 2010, 2012; Robbins *et al*, 2013).

4.3 Results

4.3.1 Dopaminergic markers

4.3.1.1 D1 receptor

A 2-way ANOVA (drug treatment x housing condition) of D1 receptor protein expression in the PFC of male rats showed no significant effects for drug treatment [$F_{1,12} = 0.127$, $p = 0.727$], or housing condition [$F_{1,12} = 0.056$, $p = 0.816$] and no interaction between these variables was present [$F_{1,12} = 0.081$, $p = 0.781$] (Figure 4.3A). A significant main effect for hemisphere was observed [$F_{1,12} = 15.338$, $p = 0.002$] with a finding of higher D1 receptor protein expression in the left PFC (1.007 ± 0.092) as compared to the right PFC (0.671 ± 0.029) (Figure 4.3C). No interactions were observed between hemisphere and any other variable. When the hemispheres were subsequently analyzed separately, no significant main effects were observed in any variable, in either hemisphere (see Table 4.1A).

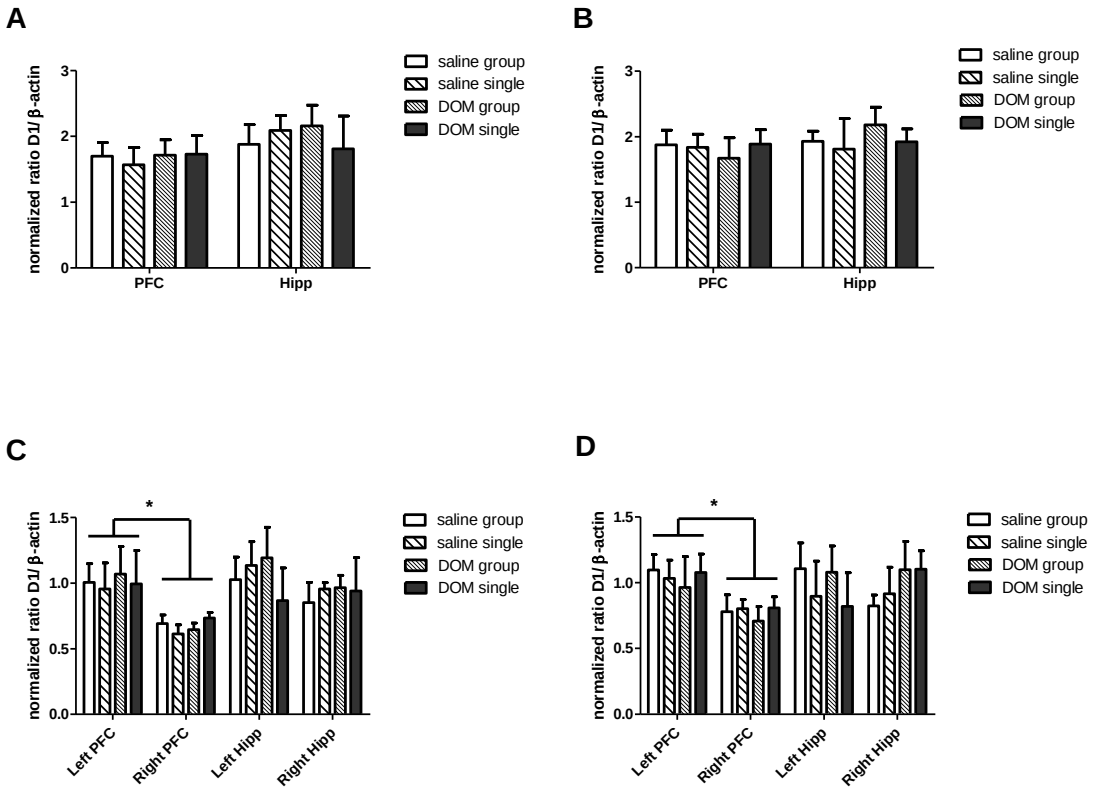

Figure 4.3 D1 receptor protein expression in the combined hemispheres of PFC and hippocampus of male (A) and female (B) rats treated neonatally with DOM or saline and housed in isolation or groups of 4. Panels C and D show D1 receptor expression values broken down by hemisphere for males (C) and females (D). * Indicates a p value of < 0.05.

Table 4.1 Results of statistical analyses for D1 receptor protein expression in the prefrontal cortex (A) and hippocampus (B) of male and female adult rats treated neonatally with DOM or saline and reared in social housing or in isolation.

A. Prefrontal cortex

Hem.	Left		Right	
Sex	Male	Female	Male	Female
Drug	$F_{1,12} = 0.060$ $p = 0.810$	$F_{1,12} = 0.071$ $p = 0.794$	$F_{1,12} = 0.427$ $p = 0.526$	$F_{1,12} = 0.108$ $p = 0.748$
Housing	$F_{1,12} = 0.094$ $p = 0.764$	$F_{1,12} = 0.025$ $p = 0.876$	$F_{1,12} = 0.004$ $p = 0.948$	$F_{1,12} = 0.385$ $p = 0.547$
Drug x Housing	$F_{1,12} = 0.004$ $p = 0.952$	$F_{1,12} = 0.295$ $p = 0.597$	$F_{1,12} = 2.016$ $p = 0.181$	$F_{1,12} = 0.149$ $p = 0.706$

B. Hippocampus

Hem.	Left		Right	
Sex	Male	Female	Male	Female
Drug	$F_{1,12} = 0.057$ $p = 0.815$	$F_{1,12} = 0.049$ $p = 0.829$	$F_{1,12} = 0.102$ $p = 0.755$	$F_{1,12} = 1.890$ $p = 0.194$
Housing	$F_{1,12} = 0.261$ $p = 0.619$	$F_{1,12} = 1.022$ $p = 0.332$	$F_{1,12} = 0.067$ $p = 0.800$	$F_{1,12} = 0.078$ $p = 0.785$
Drug x Housing	$F_{1,12} = 1.051$ $p = 0.326$	$F_{1,12} = 0.011$ $p = 0.917$	$F_{1,12} = 0.166$ $p = 0.691$	$F_{1,12} = 0.067$ $p = 0.800$

In females, no significant effects for drug treatment [$F_{1,12} = 0.099$, $p = 0.758$] or housing condition [$F_{1,12} = 0.131$, $p = 0.724$] were observed and no interaction between these variables was present [$F_{1,12} = 0.275$, $p = 0.610$] (Figure 4.3B). As with the males, a significant main effect for hemisphere was found [$F_{1,12} = 19.753$, $p = 0.001$] with D1 receptor protein expression being greater in the left PFC (1.042 ± 0.075) than the right PFC (0.775 ± 0.046) (Figure 4.3D). However, no interactions were observed between hemisphere and any other variable and when the hemispheres were subsequently analyzed separately, no significant main effects were observed in any variable, in either hemisphere (Table 4.1A).

In the hippocampus, a 2-way ANOVA (drug treatment x housing condition) of D1 receptor protein expression revealed no significant effects in the males for drug treatment [$F_{1,12} < 0.001$, $p = 0.999$], or housing condition [$F_{1,12} = 0.037$, $p = 0.851$] and no interaction between these variables was present [$F_{1,12} = 0.640$, $p = 0.439$]. No significant main effect for hemisphere was observed [$F_{1,12} = 4.096$, $p = 0.066$], although the value was approaching significance and had the same trend observed in the analyses of D1 expression in the PFC (see above) whereby D1 receptor expression appeared greater in the left hippocampus (1.056 ± 0.010) as compared to the right hippocampus (0.929 ± 0.071) at least in 3 of the 4 groups (Figure 4.3C). No interactions were observed between hemisphere and any other variable. When the hemispheres were subsequently analyzed separately in order to remain consistent with previous analyses, no significant main effects were observed in any variable in the left or right hemisphere (Table 4.1B).

In females, no significant effects were found for drug treatment [$F_{1,12} = 0.372$, $p = 0.554$] or housing condition [$F_{1,12} = 0.400$, $p = 0.539$] and no interaction between these

variables was present [$F_{1,12}$ = 0.053, p = 0.822]. Furthermore, no significant effect was observed for hemisphere [$F_{1,12}$ = 0.005, p = 0.943] and no interactions were found between hemisphere and any of the other variables (Figure 4.3B). In order to remain consistent with previous analyses, the hemispheres were subsequently analyzed separately (Figure 4.3D) and no significant main effects were observed in any variable in either the left or right hemispheres, nor were any interactions present (Table 4.1B).

4.3.1.2 D2 receptor

A 2-way ANOVA (drug treatment x housing condition) of D2 receptor protein expression in the PFC of males revealed no significant effects for drug treatment [$F_{1,12}$ = 0.850, p = 0.375], or housing condition [$F_{1,12}$ = 0.575, p = 0.463] and no interaction between these variables was present [$F_{1,12}$ = 0.104, p = 0.752]. No significant main effect for hemisphere was observed [$F_{1,12}$ = 0.045, p = 0.836] and no interactions were seen between hemisphere and any other variable (Figure 4.4A). When the hemispheres were subsequently analyzed separately (Figure 4.4C), no significant effects were observed in any variable, in either hemisphere (see Table 4.2A).

In females, no significant effects for drug treatment [$F_{1,12}$ = 0.031, p = 0.864] or housing condition [$F_{1,12}$ = 0.004, p = 0.951] were found and no interaction between these variables was present [$F_{1,12}$ = 0.144, p = 0.711]. Likewise, no main effect for hemisphere was found [$F_{1,12}$ = 0.121, p = 0.733] and no interactions were observed between hemisphere and any other variable (Figure 4.4B). When the hemispheres were subsequently analyzed separately (Figure 4.4D), no significant effects were observed in any variable, in either hemisphere (see Table 4.2A).

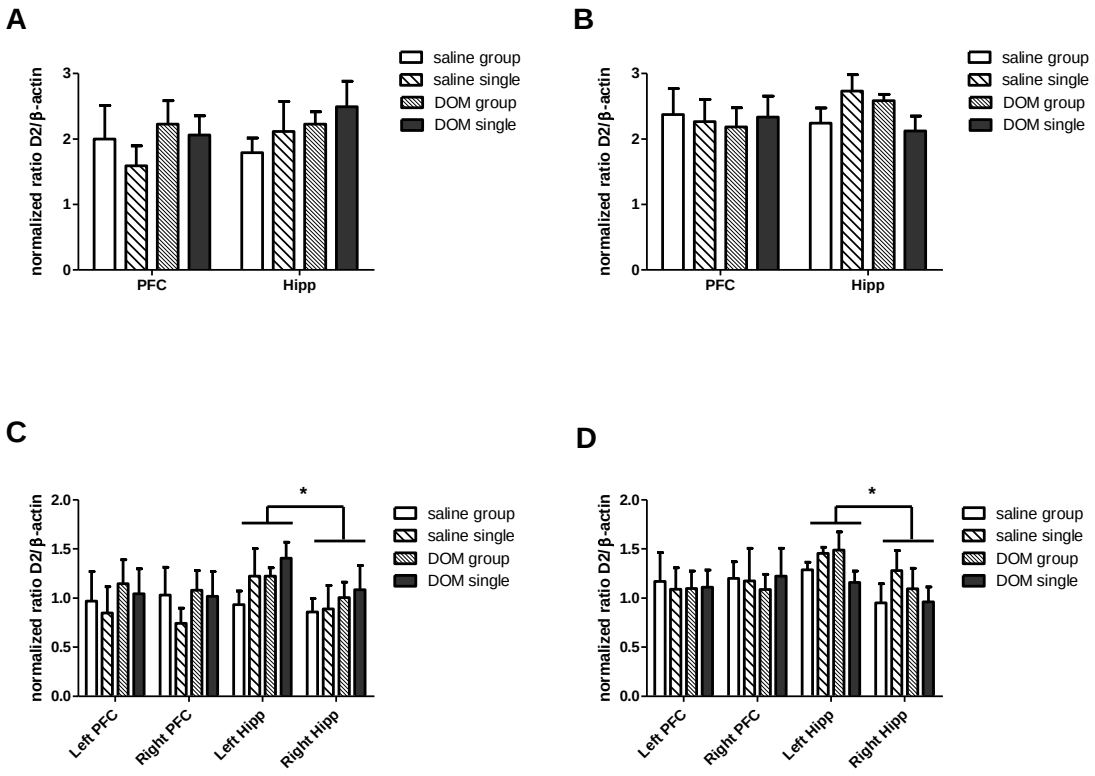

Figure 4.4 D2 receptor protein expression in the combined hemispheres of PFC and hippocampus of male (A) and female (B) rats treated neonatally with DOM or saline and housed in isolation or groups of 4. Panels C and D show D1 receptor expression values broken down by hemisphere for males (C) and females (D). * Indicates a p value of < 0.05.

Table 4.2 Results of statistical analyses for D2 receptor expression in the prefrontal cortex (A) and hippocampus (B) of male and female adult rats treated neonatally with DOM or saline and reared in social housing or in isolation.

A. Prefrontal cortex

Hem.	Left		Right	
Sex	Male	Female	Male	Female
Drug	$F_{1,12} = 0.488$ $p = 0.498$	$F_{1,12} = 0.015$ $p = 0.906$	$F_{1,12} = 0.506$ $p = 0.491$	$F_{1,12} = 0.018$ $p = 0.895$
Housing	$F_{1,12} = 0.174$ $p = 0.684$	$F_{1,12} = 0.025$ $p = 0.878$	$F_{1,12} = 0.592$ $p = 0.456$	$F_{1,12} = 0.018$ $p = 0.895$
Drug x Housing	$F_{1,12} = 0.001$ $p = 0.972$	$F_{1,12} = 0.047$ $p = 0.831$	$F_{1,12} = 0.245$ $p = 0.630$	$F_{1,12} = 0.109$ $p = 0.747$

B. Hippocampus

Hem.	Left		Right	
Sex	Male	Female	Male	Female
Drug	$F_{1,12} = 1.698$ $p = 0.217$	$F_{1,12} = 0.162$ $p = 0.694$	$F_{1,12} = 0.704$ $p = 0.418$	$F_{1,12} = 0.214$ $p = 0.652$
Housing	$F_{1,12} = 1.712$ $p = 0.215$	$F_{1,12} = 0.469$ $p = 0.507$	$F_{1,12} = 0.077$ $p = 0.786$	$F_{1,12} = 0.257$ $p = 0.621$
Drug x Housing	$F_{1,12} = 0.091$ $p = 0.768$	$F_{1,12} = 4.236$ $p = 0.062$	$F_{1,12} = 0.015$ $p = 0.905$	$F_{1,12} = 1.458$ $p = 0.250$

Analysis of D2 receptor expression in the hippocampus revealed a similar pattern to the PFC. A 2-way ANOVA (drug treatment x housing condition) of D2 receptor expression in males revealed no significant effects for drug treatment [$F_{1,12} = 1.458$, $p = 0.251$], or housing condition [$F_{1,12} = 0.765$, $p = 0.399$] and no interaction between these variables was present [$F_{1,12} = 0.008$, $p = 0.930$] (Figure 4.4A). A significant main effect for hemisphere was observed [$F_{1,12} = 6.576$, $p = 0.025$], with more D2 receptor protein being found in the left hemisphere (1.196 ± 0.092) as compared to the right hemisphere (0.959 ± 0.093) (Figure 4.4C). No interactions were observed between hemisphere and any other variable. When the hemispheres were subsequently analyzed separately, no significant main effects were observed in any variable in the left or right hemisphere (Table 4.2B).

In female rats, no significant effects were seen for drug treatment [$F_{1,12} = 0.416$, $p = 0.531$] or housing condition [$F_{1,12} = 0.005$, $p = 0.947$] although a significant interaction was found between drug treatment and housing condition [$F_{1,12} = 5.072$, $p = 0.044$]. A significant main effect was also found for hemisphere [$F_{1,12} = 5.382$, $p = 0.039$] with a greater amount of D2 receptor protein being found in the left hemisphere (1.348 ± 0.064) compared to the right hemisphere (1.072 ± 0.092) (Figure 4.4D). No interactions were found between hemisphere and any other variable. When the hemispheres were subsequently analyzed separately, no significant main effects were observed in any variable in either the left or right hemispheres, nor were any interactions present (Table 4.2B).

4.3.1.3 Tyrosine hydroxylase

Analyses of TH protein expression in the PFC of male rats revealed no significant effect for drug treatment [$F_{1,12} = 3.332$, $p = 0.093$], or housing condition [$F_{1,12} = 0.510$, $p = 0.489$] and no interaction between these variables was present [$F_{1,12} = 1.025$, $p = 0.331$]. No significant main effect for hemisphere was observed [$F_{1,12} = 0.692$, $p = 0.442$] and no interactions were found between hemisphere and any other variable (Figure 4.5A). When the hemispheres were subsequently analyzed separately, no significant effects were observed in any variable, in either hemisphere (see Table 4.3A) although there was a consistent trend of increased TH protein in the PFCs of DOM treated rats, particularly in the single housed animals (see Figure 4.5C).

Females showed no significant effects for drug treatment [$F_{1,12} = 0.910$, $p = 0.359$] or housing condition [$F_{1,12} = 0.055$, $p = 0.819$] and no interaction between these variables was present [$F_{1,12} = 0.034$, $p = 0.856$]. Likewise, no main effect for hemisphere was found [$F_{1,12} = 2.500$, $p = 0.140$] and no interactions were observed between hemisphere and any other variable (Figure 4.5B). In order to remain consistent with the other analyses in this study, hemispheres were subsequently analyzed separately (Figure 4.5D). No significant effects were observed in any variable, in either hemisphere (see Table 4.3A).

Analysis of TH protein expression in the hippocampus revealed no significant effects in males for drug treatment [$F_{1,12} = 0.171$, $p = 0.687$], or housing condition [$F_{1,12} = 0.157$, $p = 0.699$] and no interaction between these variables was present [$F_{1,12} = 0.015$, $p = 0.906$]. No significant main effect for hemisphere was observed [$F_{1,12} = 3.019$, $p = 0.108$] and no interactions between hemisphere and any other variable were found (Figure 4.5A). When the hemispheres were subsequently analyzed separately

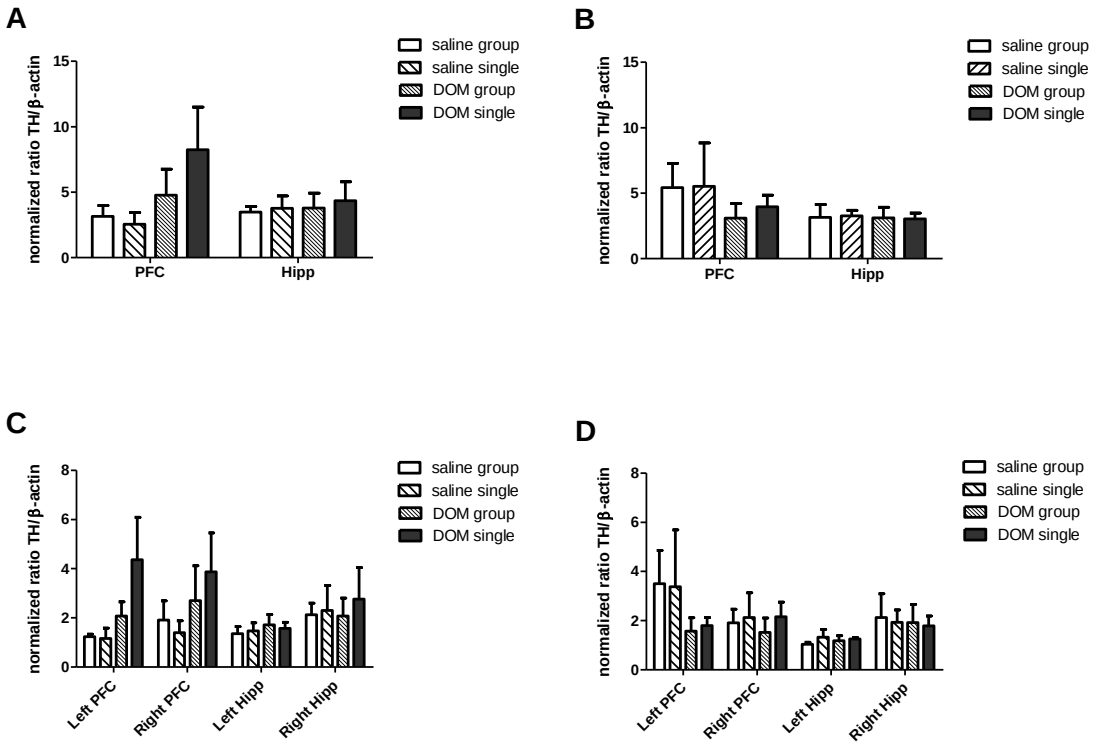

Figure 4.5 Tyrosine hydroxylase expression in the combined hemispheres of PFC and hippocampus of male (A) and female (B) rats treated neonatally with DOM or saline and housed in isolation or groups of 4. Panels C and D show TH expression values broken down by hemisphere for males (C) and females (D).

Table 4.3 Results of statistical analyses for tyrosine hydroxylase protein expression in the prefrontal cortex (A) and hippocampus (B) of male and female adult rats treated neonatally with DOM or saline and reared in social housing or in isolation.

A. Prefrontal cortex

Hem.	Left		Right	
Sex	Male	Female	Male	Female
Drug	$F_{1,12} = 4.674$ $p = 0.052$	$F_{1,12} = 1.629$ $p = 0.226$	$F_{1,12} = 2.008$ $p = 0.182$	$F_{1,12} = 0.069$ $p = 0.797$
Housing	$F_{1,12} = 1.399$ $p = 0.260$	$F_{1,12} = 0.001$ $p = 0.972$	$F_{1,12} = 0.079$ $p = 0.783$	$F_{1,12} = 0.364$ $p = 0.558$
Drug x Housing	$F_{1,12} = 1.598$ $p = 0.230$	$F_{1,12} = 0.015$ $p = 0.904$	$F_{1,12} = 0.538$ $p = 0.477$	$F_{1,12} = 0.086$ $p = 0.774$

B. Hippocampus

Hem.	Left		Right	
Sex	Male	Female	Male	Female
Drug	$F_{1,12} = 0.503$ $p = 0.492$	$F_{1,12} = 0.031$ $p = 0.863$	$F_{1,12} = 0.048$ $p = 0.831$	$F_{1,12} = 0.063$ $p = 0.805$
Housing	$F_{1,12} = 0.001$ $p = 0.971$	$F_{1,12} = 0.870$ $p = 0.369$	$F_{1,12} = 0.215$ $p = 0.651$	$F_{1,12} = 0.053$ $p = 0.822$
Drug x Housing	$F_{1,12} = 0.142$ $p = 0.713$	$F_{1,12} = 0.367$ $p = 0.556$	$F_{1,12} = 0.074$ $p = 0.791$	$F_{1,12} = 0.002$ $p = 0.966$

(Figure 4.5C), no significant main effects were observed in any variable in the left or right hemisphere (Table 4.3B).

No significant effects were seen in the hippocampus of female rats for drug treatment [$F_{1,12} = 0.038$, $p = 0.848$], housing condition [$F_{1,12} = 0.001$, $p = 0.975$] nor was an interaction between those two variables present [$F_{1,12} = 0.015$, $p = 0.904$]. A significant main effect was not found for hemisphere [$F_{1,12} = 4.259$, $p = 0.061$] and no interactions were found between hemisphere and any other variable (Figure 4.5B). When the hemispheres were subsequently analyzed separately (Figure 4.5D), no significant main effects were observed in any variable in either the left or right hemispheres, nor were any interactions present (Table 4.3B).

4.3.2 GABA markers

4.3.2.1 GAD65

Analyses of GAD65 protein expression in the PFC of males revealed no significant effects for drug treatment [$F_{1,12} = 0.015$, $p = 0.905$], or housing condition [$F_{1,12} = 0.285$, $p = 0.603$] and no interaction between these variables was present [$F_{1,12} = 0.606$, $p = 0.451$] (Figure 4.6A). No significant main effect for hemisphere was observed although the value was approaching significance [$F_{1,12} = 4.510$, $p = 0.055$] with a trend of higher GAD65 protein expression in the left hemisphere (0.867 ± 0.089) as compared to the right hemisphere (0.734 ± 0.087) (Figure 4.6C). No interactions were observed between hemisphere and any other variable. When hemispheres were further analyzed separately, no significant main effects were observed in any variable, in either hemisphere (see Table 4.4A).

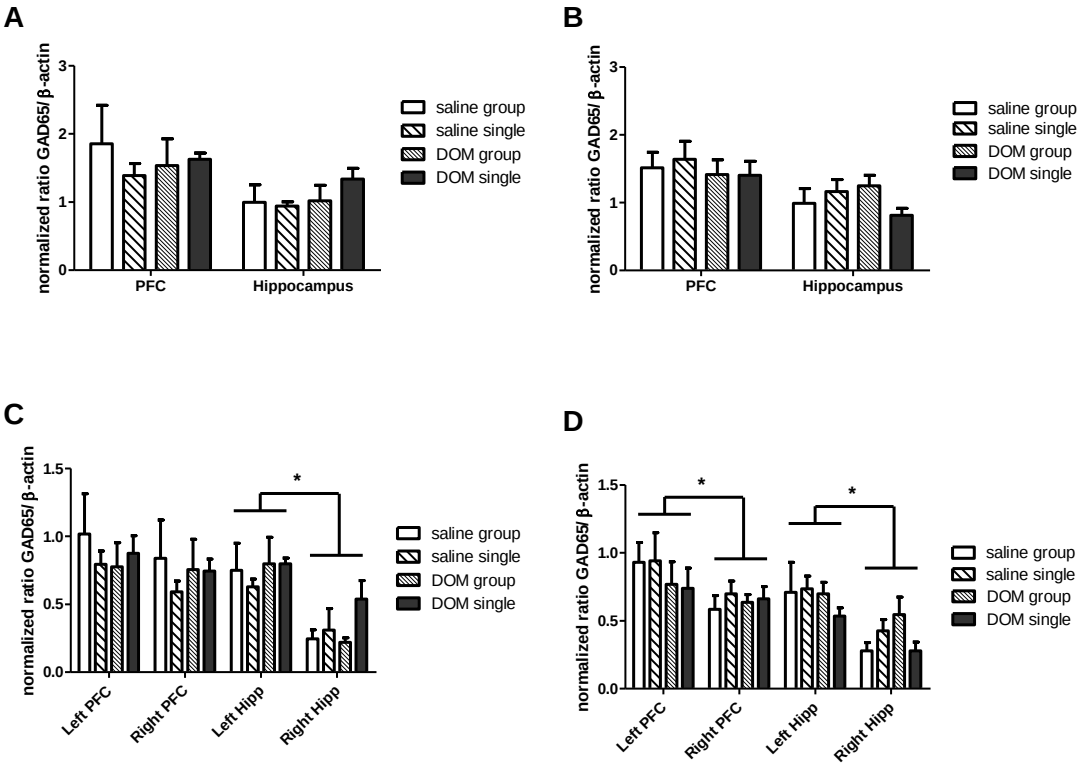

Figure 4.6 GAD65 protein expression in the combined hemispheres of PFC and hippocampus of male (A) and female (B) rats treated neonatally with DOM or saline and housed in isolation or groups of 4. Panels C and D show GAD65 expression values broken down by hemisphere for males (C) and females (D). * Indicates a p value of < 0.05.

Table 4.4 Results of statistical analyses for GAD 65 protein expression in the prefrontal cortex (A) and hippocampus (B) of male and female adult rats treated neonatally with DOM or saline and reared in social housing or in isolation. * indicates a significant main effect or an interaction for that/those variable(s) ($p < 0.05$).

A. Prefrontal cortex

Hem.	Left		Right	
Sex	Male	Female	Male	Female
Drug	$F_{1,12} = 0.170$ $p = 0.687$	$F_{1,12} = 1.163$ $p = 0.302$	$F_{1,12} = 0.034$ $p = 0.856$	$F_{1,12} = 0.007$ $p = 0.933$
Housing	$F_{1,12} = 0.103$ $p = 0.754$	$F_{1,12} = 0.003$ $p = 0.959$	$F_{1,12} = 0.475$ $p = 0.504$	$F_{1,12} = 0.639$ $p = 0.439$
Drug x Housing	$F_{1,12} = 0.715$ $p = 0.414$	$F_{1,12} = 0.016$ $p = 0.901$	$F_{1,12} = 0.388$ $p = 0.545$	$F_{1,12} = 0.264$ $p = 0.617$

B. Hippocampus

Hem.	Left		Right	
Sex	Male	Female	Male	Female
Drug	$F_{1,12} = 0.558$ $p = 0.469$	$F_{1,12} = 0.644$ $p = 0.438$	$F_{1,12} = 1.401$ $p = 0.259$	$F_{1,12} = 0.442$ $p = 0.519$
Housing	$F_{1,12} = 0.174$ $p = 0.684$	$F_{1,12} = 0.286$ $p = 0.602$	$F_{1,12} = 4.999$ $p = 0.045$*	$F_{1,12} = 0.442$ $p = 0.519$
Drug x Housing	$F_{1,12} = 0.147$ $p = 0.684$	$F_{1,12} = 0.527$ $p = 0.482$	$F_{1,12} = 2.179$ $p = 0.166$	$F_{1,12} = 5.286$ $p = 0.040$*

In females, there were no significant effects for drug treatment [$F_{1,12} = 0.567$, $p = 0.466$] or housing condition [$F_{1,12} = 0.070$, $p = 0.795$] and no interaction between these variables was present [$F_{1,12} = 0.082$, $p = 0.779$] (Figure 4.6B). A significant main effect for hemisphere was found [$F_{1,12} = 8.561$, $p = 0.013$] with GAD65 protein expression being greater in the left hemisphere (0.846 ± 0.079) versus the right hemisphere (0.646 ± 0.041) (Figure 4.6D), however, no interactions were observed between hemisphere and any other variable. When the hemispheres were subsequently analyzed separately, no significant main effects were observed in any variable, in either hemisphere (see Table 4.4A).

Analysis of GAD65 protein expression in the hippocampus revealed no significant effects in males for drug treatment [$F_{1,12} = 1.161$, $p = 0.302$], or housing condition [$F_{1,12} = 0.459$, $p = 0.511$] and no interaction between these variables was present [$F_{1,12} = 0.924$, $p = 0.355$] (Figure 4.6A). A significant main effect for hemisphere was observed [$F_{1,12} = 37.284$, $p < 0.001$] with higher GAD65 protein expression in the left hippocampus (0.744 ± 0.067) compared to the right hippocampus (0.328 ± 0.050) (Figure 4.6C). No interactions between hemisphere and any other variable were present. When the hemispheres were subsequently analyzed separately, no significant main effects were observed in any variable in the left hemisphere (Table 4.1B) but a significant main effect for housing condition was found in the right hemisphere ($F_{1,12} = 4.999$, $p = 0.045$]. When this effect was investigated further using t-tests, no significant differences were observed among any of the groups (Table 4.4B). Interestingly, single housed males showed a general trend of increased GAD65 protein in the hippocampus of DOM treated rats compared to saline treated rats.

Females showed no significant effects for drug treatment [$F_{1,12}$ = 0.070, p = 0.796] or housing condition [$F_{1,12}$ = 0.586, p = 0.459] and no interaction between these variables was present [$F_{1,12}$ = 3.171, p = 0.100] (Figure 4.6B). A significant main effect for hemisphere was found [$F_{1,12}$ = 15.395, p = 0.002] with GAD65 protein expression being greater in the left hippocampus (0.670 ± 0.044) versus the right hippocampus (0.381 ± 0.035) (Figure 4.6D). However, no interactions were observed between hemisphere and any other variable. When the hemispheres were subsequently analyzed separately, no significant main effects were observed in any variable in the left hemisphere (Table 4.4B) but a significant interaction between drug treatment and housing condition was found in the right hemisphere [$F_{1,12}$ = 5.286, p = 0.040]. When this effect was investigated further using t-tests, no significant differences were observed among any of the groups although there was a consistent trend in the single housed rats whereby DOM treated rats showed less GAD65 protein than saline treated rats. Additionally, in rats that received DOM treatment there was a general trend for single housed rats to have less hippocampal GAD65 than group housed rats.

4.3.2.2 GAD67

Analyses of GAD67 protein expression in the PFC of male rats revealed no significant effects for drug treatment [$F_{1,12}$ = 0.232, p = 0.639], or housing condition [$F_{1,12}$ = 0.178, p = 0.681] and no interaction between these variables was present [$F_{1,12}$ = 0.018, p = 0.896] (Figure 4.7A). A significant main effect for hemisphere was observed [$F_{1,12}$ = 10.402, p = 0.007] with a finding of higher GAD67 protein expression in the left PFC (0.520 ± 0.073) compared to the right PFC (0.342 ± 0.056) (Figure 4.7C). No interactions were observed between hemisphere and any other variable. When the

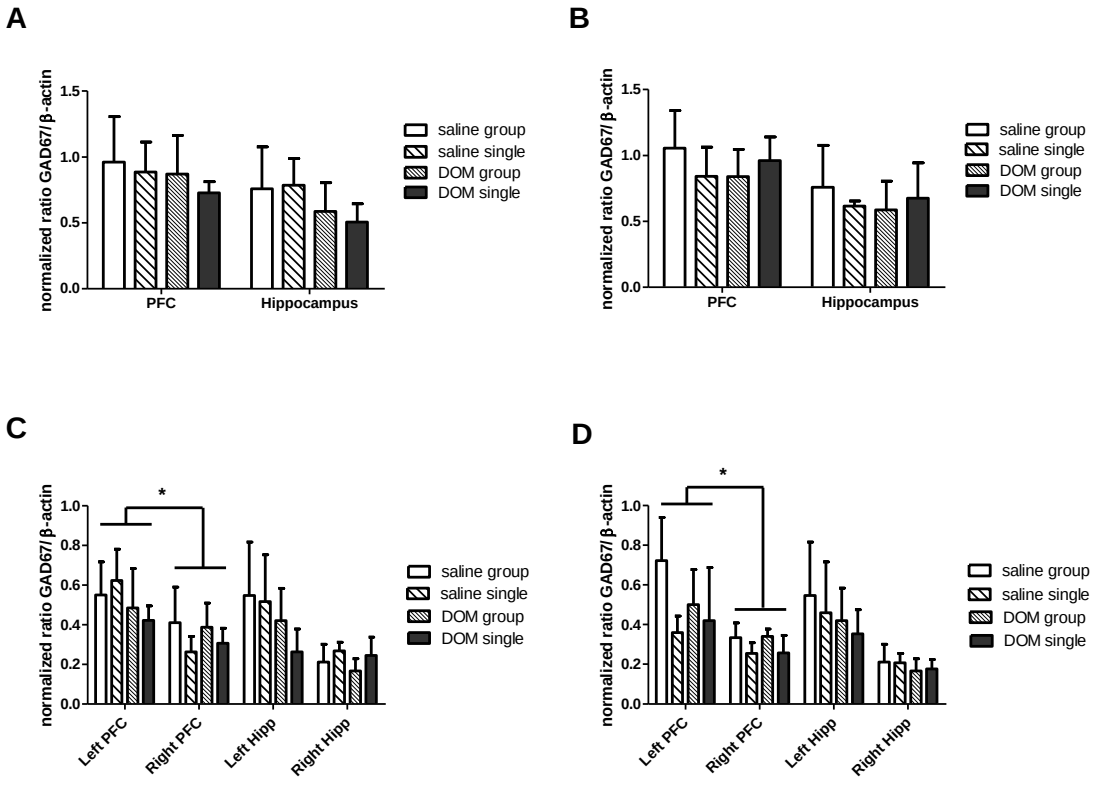

Figure 4.7 GAD 67 protein expression in the combined hemispheres of PFC and hippocampus of male (A) and female (B) rats treated neonatally with DOM or saline and housed in isolation or groups of 4. Panels C and D show GAD67 expression values broken down by hemisphere for males (C) and females (D). * Indicates a p value of < 0.05.

hemispheres were subsequently analyzed separately, no significant main effects were observed in any variable, in either hemisphere (see Table 4.5A).

In females no significant effects were seen for drug treatment [$F_{1,12} = 0.046$, $p = 0.834$] or housing condition [$F_{1,12} = 0.043$, $p = 0.840$] and no interaction between these variables was present [$F_{1,12} = 0.056$, $p = 0.474$] (Figure 4.7B). A main effect for hemisphere was found [$F_{1,12} = 6.967$, $p = 0.022$] with GAD 67 protein expression being greater in the left PFC (0.571 ± 0.061) versus the right PFC (0.354 ± 0.023) (Figure 4.7D). However, no interactions were observed between hemisphere and any other variable and when the hemispheres were subsequently analyzed separately, no significant main effects were observed in any variable, in either hemisphere (see Table 4.5A).

In the hippocampus, analysis of GAD67 protein expression revealed no significant effects in males for drug treatment [$F_{1,12} = 0.056$, $p = 0.817$], or housing condition [$F_{1,12} = 0.014$, $p = 0.912$] and no interaction between these variables was present [$F_{1,12} = 0.240$, $p = 0.633$] (Figure 4.7A). No significant main effect for hemisphere was observed [$F_{1,12} = 4.129$, $p = 0.065$] but the same trend of higher GAD67 protein in the left hippocampus (0.437 ± 0.096) compared to the right hippocampus (0.223 ± 0.035) was present (Figure 4.7C). No interactions were observed between hemisphere and any other variable. When the hemispheres were subsequently analyzed separately, no significant main effects were observed in any variable in the left or right hemisphere.

Table 4.5 Results of statistical analyses for GAD 67 protein expression in the prefrontal cortex (A) and hippocampus (B) of male and female adult rats treated neonatally with DOM or saline and reared in social housing or in isolation.

A. Prefrontal cortex

Hem.	Left		Right	
Sex	Male	Female	Male	Female
Drug	$F_{1,12} = 0.728$ $p = 0.410$	$F_{1,12} = 1.179$ $p = 0.679$	$F_{1,12} = 0.006$ $p = 0.939$	$F_{1,12} = 0.176$ $p = 0.682$
Housing	$F_{1,12} = 0.001$ $p = 0.972$	$F_{1,12} = 0.188$ $p = 0.673$	$F_{1,12} = 0.880$ $p = 0.367$	$F_{1,12} = 0.217$ $p = 0.650$
Drug x Housing	$F_{1,12} = 0.187$ $p = 0.673$	$F_{1,12} = 0.602$ $p = 0.453$	$F_{1,12} = 0.075$ $p = 0.789$	$F_{1,12} = 0.113$ $p = 0.743$

B. Hippocampus

Hem.	Left		Right	
Sex	Male	Female	Male	Female
Drug	$F_{1,12} = 0.026$ $p = 0.874$	$F_{1,12} = 0.312$ $p = 0.587$	$F_{1,12} = 0.083$ $p = 0.778$	$F_{1,12} = 2.501$ $p = 0.140$
Housing	$F_{1,12} = 0.197$ $p = 0.665$	$F_{1,12} = 0.016$ $p = 0.903$	$F_{1,12} = 0.798$ $p = 0.389$	$F_{1,12} = 2.757$ $p = 0.123$
Drug x Housing	$F_{1,12} = 0.194$ $p = 0.668$	$F_{1,12} < 0.001$ $p = 0.989$	$F_{1,12} = 0.093$ $p = 0.765$	$F_{1,12} = 4.023$ $p = 0.068$

In females, no significant effects were seen for drug treatment [$F_{1,12} = 0.003$, $p = 0.960$] or housing condition [$F_{1,12} = 0.480$, $p = 0.502$] and no interaction between these variables was present [$F_{1,12} = 0.473$, $p = 0.505$]. Furthermore, no significant effect was observed for hemisphere [$F_{1,12} = 2.618$, $p = 0.132$] and no interactions were found between hemisphere and any of the other variables (Figure 4.7B). In order to remain consistent with previous analyses, the hemispheres were subsequently analyzed separately (Figure 4.7D) and no significant main effects were observed in any variable in either the left or right hemispheres, nor were any interactions present (Table 4.5B).

4.4 Discussion

In contrast to the abundant behavioural effects described in Chapter 3, there was very little indication of change in any of the protein markers assessed in this study. No statistically significant differences in D1 receptor protein, D2 receptor protein or TH protein were observed among any experimental group, in either sex. Interestingly, there was a tendency for increased TH protein in the PFCs of male DOM treated rats which was particularly noticeable in the single housed males, but this was not statistically significant. As indicated in Figure 4.5A/C, this increased TH was accompanied by a large degree of variability. When this effect was investigated further by looking at the individual data points, it was clear that this increased mean and large variability was not due to a single outlier, with all animals in the DOM treated/single housed group demonstrating very different amounts of TH in the PFC. This finding of large variability within groups was shown to a lesser extent in other markers and could indicate a potential explanation for the lack of significant differences observed in this study. It could be that differences are present in individual animals but not consistently within all

animals in a given experimental group. In other words, an effect that is present in some individual animals is being hidden by those animals that did not show a change when we combine all animals in a given group together for statistical analysis. This idea is expanded upon further in Appendix C.

Another interesting trend was observed with the GABAergic markers. As with the dopaminergic markers, no significant differences were found between experimental groups in either GAD65 or GAD67 protein. However, male rats did show a trend of increased GAD65 in the right hippocampus of DOM treated rats that were housed in isolation as compared to DOM treated rats that were housed in groups (Figure 4.6 A/C) and in fact this difference would have been statistically significant if the 1-tailed value was used. The opposite trend was observed in females where DOM treated, single housed rats appeared to have less GAD65 protein in the right hippocampus compared to DOM treated rats that were group housed (Figure 4.6 B/D) and again, this difference would be statistically significant if 1-tailed values were used. It is interesting that these trends were present in the right hippocampus alone, as the GAD67 data indicates the trends of lowered GAD67 protein due to DOM treatment are found primarily in the left PFC and left hippocampus of both males and females (Figure 4.7).

The lack of significant findings in this study is surprising given that these neurotransmitter systems are known in the literature to be associated with the behavioural changes observed in Chapter 3 (see section 4.1). However, while both the DA and GABA systems are known to be involved, these are very complex behaviours which presumably involve many brain areas and multiple neurotransmitter systems. While the reasoning for investigating these markers was appropriate, it is certainly possible that it is changes to other brain areas that are responsible for the significant

behavioural effects observed in Chapter 3. For example, DA transmission in the NAc is thought to play an important role in LI and therefore could be an area for future study using this model (Han *et al*, 2012; Shao *et al*, 2009; Weiner, 2003). It is also possible that it is small alterations in a variety of brain areas and the resulting changes in how those areas interact with each other that are responsible for the behavioural effects previously observed. Another possibility is that these systems have changed, even within the areas studied, but that the investigation of other markers would have shown better results. A recent study by (Pehrson *et al*, 2013) found that attentional processing was impaired as a result of a pharmacological reduction in $GABA_A$ receptor functioning but was unaffected by a reduction in GAD67. This finding suggests that it may not be the concentration of protein that is important to the role of GABA in attentional processing, but rather the functional activity of the system.

It is also possible that there are changes in these markers within these brain areas, but they were not detected. One issue with protein analysis by Western blot, and indeed, a possible explanation for why we did not observe changes, is that only somewhat larger brain areas can be investigated because of the amount of protein required. In this experiment the hippocampus was taken as one structure (although right and left hemispheres were separated). Also in the case of the PFC, the entire PFC was taken and homogenized. While processing the tissue in this manner is necessary for Western blotting, it does have limitations and makes it unlikely that differences localized to only some subsections of a structure might be detected. While past studies using the neonatal DOM model have suggested changes to both the DA and GABA systems, the changes have often been slight (often region specific, sex specific or hemisphere specific) (Adams-Marriott, 2009; Gill *et al*, 2010, 2012; Robbins *et al*, 2013). Differences in

experimental design are another possible explanation for why we did not observe those same changes. For example, we were not able to look at the ventral versus dorsal hippocampus, nor were we able to assess the various subfields of the PFC. Therefore, if very small differences existed in only a small region of the brain, it is conceivable that this difference would no longer be detectable; the effect having been diluted by the other brain tissue during homogenization and processing.

It is important to recognize that measuring protein concentrations (as was done in this study) does not provide conclusive information about the activity of those systems as it does not measure that activity itself. It is theoretically possible for the total amount of receptor protein to remain the same, but for the functional properties of those receptors to be altered (e.g. posttranslational modification). It is also possible for other aspects of neurotransmission within a system to have changed (e.g. neurotransmitter synthesis, reuptake, degradation) while the receptor concentrations remain the same. Along this same line of thinking, it is interesting to note that in this study it appears that D_1 and D_2 receptor protein levels are more consistent across the experimental groups, while greater group differences are observed for TH, GAD65 and GAD67, which are implicated in the synthesis of DA and GABA respectively rather than the receptors themselves. Thus, it might be helpful in future studies to use other methods (e.g. high-performance liquid chromatography) to measure activity in a more direct manner. Additionally, it could be argued that the variability within groups appears to be higher in TH, GAD65 and GAD67 as compared to D_1 and D_2, lending further support for the idea that perhaps changes in these marker have occurred, but that they have been masked by the high degree of variability within some of the treatment groups.

Interestingly, the most consistent finding of this study was significant hemispheric differences in the overall amount of protein expression. These differences were found in at least one brain area for all protein markers and always revealed higher protein in the left hemisphere, except in the case of TH, which was higher in the right hemisphere. The issue of lateral asymmetry of neurotransmitter systems in the brain has been a topic of debate. Lateralization is well established in animals, with results in healthy rats including increased GAD in the left striatum vs. the right striatum (Guarneri *et al*, 1985), increased D2 in the left striatum vs. the right striatum (Schneider *et al*, 1982) and decreased D1, D2 and DA in the left cortex vs. the right cortex (Nowak, 1989). With humans, some post-mortem studies on the brains of healthy human subjects indicate no differences between the hemispheres (Rossor *et al*, 1980), while other studies suggest that differences do exist (Glick *et al*, 1982). A study by Glick *et al*, (1982) looked at a number of brain areas not assessed in this study (in caudate, putamen and globus pallidus), but never-the-less, indicated a number of interesting findings including that DA was greater in the left hemisphere, that there was a significant trend for brains with lower overall DA levels to be left-biased and those brains with overall higher DA levels to be right biased. They also concluded that many of their finding had been previously reported in rats, indicating that rodents and humans may share similar right-left asymmetries in neurotransmitters (Glick *et al*, 1982).

A number of lateralization differences also have been found in both people with schizophrenia (DeLisi *et al*, 1991) as well as in current animals models (Hikida *et al*, 2007; Pletnikov *et al*, 2008), with the left hemisphere often being affected to a greater extent than the right. Specifically (Reynolds, 1983) found increased DA in the left amygdala vs. the right amygdala of people with schizophrenia.

Regarding the specific finding of higher proteins for all markers in the left hemisphere as compared with the right (except in the case of TH) a study by (Zaborszky and Vadasz, 2001) used a series of genetic mouse models known to express low, medium and high amounts of TH activity. Using these mice, they investigated TH and DA receptors in the mesencephalicc DA system and found an inverse relationship between TH and the number of DA neurons. This is intriguing, as all markers in the study described in this chapter were higher in the left hemisphere (including D1 and D2 receptor protein) except for TH, which was higher in the right. This may indicate some sort of compensatory relationship between these markers of DA activity.

While the results of investigations into lateralization of neurotransmitter systems in the brains of both healthy human and animal subjects, as well as those in the clinical population and models are unclear and often contradictory, it does appear that hemispheric differences are not uncommon. Therefore it is perhaps not surprising that hemispheric differences were found in this study and that it only seemed unusual since carrying out separate analyses for each hemisphere seems relatively uncommon.

In conclusion, while some interesting trends were observed, no significant differences were found in the protein expression of D1 receptors, D2 receptors, TH, GAD65 or GAD67. It is possible that changes in these systems have occurred but were not observed using this experimental design (a further investigation of correlations between this data and the data from Chapter 3 is explored in Appendix C). It is also possible that changes in other neurotransmitter systems and/or other brain areas are responsible for the behavioural effects observed in Chapter 3.

4.5 References

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Chapter 5

Summary of the main results, general discussion and conclusions

5.1 Goals of the thesis

The primary purpose of the experiments described herein was to better understand how early developmental factors can have a long lasting effect on the CNS and how such alterations may contribute to understanding the development of neuropsychiatric disorders. This was accomplished by investigating the effects of both neonatal low-dose DOM treatment and social isolation rearing on behavioural measures of attentional processing and the region-specific expression of relevant protein markers in the brains of those same animals. The use of these two paradigms was previously reported to produce alterations in attentional processing in rats, and the exploration of each alone and in combination, was a novel approach to investigating the "two-hit" hypothesis of psychiatric disease.

5.2 Effect of neonatal low-dose DOM treatment and social isolation rearing on behavioural measures of attentional processing

Following the establishment of experimental protocols outlined in Chapter 2, the experiments in Chapter 3 investigated the effects of neonatal low-dose DOM treatment and social isolation rearing on LI and PPI, two behavioural measures of attentional processing. Results indicated that both behaviours were affected, but the effects observed and the degree of change varied greatly depending on the sex of the animal, the test used, and the particular measure being examined.

The results of LI testing found that neonatal DOM treatment completely abolished the significant LI effect observed in male saline-treated rats at 48 hours post-testing, regardless of housing condition. Isolation housing alone also resulted in a lack of statistically significant LI in males, although the effect was not as dramatic as that seen

in DOM treated rats. When male rats were tested again one week later, significant LI was seen only in the DOM treated rats that were single-housed and was not observed in any other experimental groups.

Very different results were obtained in female rats where both single and group-housed rats treated with DOM displayed significant LI at 48 hours, while neither of the saline treated groups did (although both groups of saline-treated female rats showed a tendency toward significant LI). When tested again one week later, no LI effects were observed in any group of female rats.

Collectively, these results suggest that both of the early life interventions used affected LI, but perhaps the effects of DOM manifest mainly in the short-term whereas social isolation produces longer lasting disruptions of LI. It was concluded that neonatal treatment with DOM is highly disruptive to the normal development of LI in male rats and to a lesser extent female rats, however the lack of a statistically significant LI effect in groups of female rats in this study may be a function of variability and small sample size.

Both neonatal DOM treatment and social isolation rearing had a profound effect on the PPI of male and to a lesser extent, female rats, but these effects were different for the various measures of PPI. Social isolation significantly lowered the amplitude of PPI in male rats which is consistent with previous literature using this model (Domeney and Feldon, 1998; Geyer *et al*, 1993; Stevens *et al*, 1997). Interestingly, while neonatal DOM treatment alone did not produce any effect on PPI amplitude, it appeared to make male rats refractory to the expected effects of social isolation on PPI. When the data were analyzed for latency (as opposed to amplitude) social isolation and DOM treatment caused an additive increase in PPI startle latency that was observed in both male and

female rats. These results indicate that neonatal DOM treatment has a strong effect on all measures of PPI assessed in this study although it appears to be countering the ability of isolation rearing to affect startle amplitude and working in an additive manner with isolation rearing to affect startle latency.

It is important to point out that the behavioural changes observed in these experiments cannot be attributed to many other factors that showed no indication of differences between the groups. For LI these other factors included the amount of water consumed during training and rebaseline, the latency to begin drinking during training and rebaseline, and the amount of water consumed in the homecage (see sections 3.3.2.1, 3.3.2.2 and 3.3.2.3). For PPI these other factors included the weight of the animals, their baseline startle level, movement during testing and their habituation (see sections, 3.3.1 and 3.3.3). Additionally, it is unlikely that the observed changes were due to differences in the ability of treated animals to hear the tone because none of the experimental groups showed differences in baseline startle behaviours, indicating they were able to hear the auditory queues without issue.

One of the consistent findings in this thesis was the differences between male and female rats. These differences are not necessarily surprising given the multitude of sex differences previously observed in the neonatal domoate model (Adams *et al*, 2009; Burt *et al*, 2008; Doucette *et al*, 2007; Gill *et al*, 2010b, 2012; Marriott *et al*, 2012; Robbins *et al*, 2013; Ryan *et al*, 2011), as well as the clinical populations these interventions are seeking to represent (Häfner, 2003; Rao and Kölsch, 2003; Salokangas *et al*, 2003). Furthermore, sex-dependent baseline differences in the behaviours in question have been observed in both healthy populations (Klosterhalfen *et al*, 2005) and in the clinical population (Kumari *et al*, 2004; Lubow *et al*, 2001). While the precise

cause of the sex differences observed in this study are unclear, one possibility could be that the neonatal DOM administration protocol affected males and females differently. Developmental variations in neurotransmitter systems thought to be implicated in these behaviours could be one possibility. For example, while the developmental shift in GABA from being excitatory (depolarizing) to inhibitory (hyperpolarizing) (see section 1.3.3.3) occurs by the second week of postnatal life, the precise age of the shift may depend on sex, with the shift potentially occurring earlier in females than in males (Galanopoulou, 2008; Nuñez and McCarthy, 2007). If female rats have already undergone this developmental shift by the time the DOM treatment begins but the males have not, it is likely that the treatment which is presumed to affect overall excitatory/inhibitory balance in the brain, will affect the sexes differently.

5.3 Effects of neonatal low-dose DOM treatment and social isolation rearing on select protein expression in the brain

Following the finding of significant behavioural alterations in Chapter 3, Chapter 4 sought to investigate protein markers of neurotransmitter systems of interest, selected based on the current understanding of the brain areas and systems involved in the observed behavioural changes, their involvement in disorders observed in the human clinical population, their role in attentional processing, and the results of previous findings in these models.

No significant differences were observed in any of the proteins measured, in either sex, in any of the brain areas, although some interesting trends were present. There was a tendency for increased TH protein expression in the PFCs of male DOM treated rats which was particularly noticeable in the single housed males. This finding is

in contrast to the study by Robbins *et al*, (2013) who found less TH in the right mPFC of DOM treated male rats as compared to saline treated male rats, however there were three major differences between the two studies. The experiments described by Robbins *et al*, (2013) quantified TH using immunohistochemistry that determined the number of cells showing TH immunoreactivity, not the concentration of TH as was measured herein. Additionally, Robbins *et al*, (2013) housed the rats in groups of 2 or 3, which could also contribute to differences observed between that study and the present work. Finally, the animals used in that study were considerably older (8-9 months of age) than the animals in the present study. The work in this thesis also found that male rats showed a trend toward increased concentrations of GAD65 in the right hippocampus of DOM treated rats that were housed in isolation as compared with DOM treated rats that were housed in groups, while the opposite effect was found in female rats (i.e. DOM treated, single housed females appeared to have less GAD65 protein in the right hippocampus compared to DOM treated females that were group housed). Significant hemispheric differences in the overall amount of protein expression were also found with all markers showing higher values in the left hemisphere, with the exception of TH, which was higher in the right hemisphere. However, these differences were not group dependent.

While the results reported in Chapter 4 indicated no significant differences between groups for any of the protein markers measured, obvious visible differences in band intensity were observed through a visual inspection of the blots, suggesting considerable variability between individuals. To further investigate this observation, a correlational analysis was undertaken to compare the behavioural results described in Chapter 3, with the protein marker data from Chapter 4 for individual rats. The results of those analyses are presented in Appendix C. Results indicated a number of significant

correlations in both males and females, suggesting that neonatal DOM treatment and social isolation rearing may have lead to subtle, but functionally-relevant, changes in the DA and GABA systems of individual animals, but that these changes are sufficiently variable within groups that they mask any effect when a group is analyzed as a whole.

5.4 Assessing the interaction between neonatal low-dose DOM treatment and social isolation rearing in rats to better model aspects of human neuropsychiatric disorder

One of the original hypotheses directing the experimental work described in this thesis was that neonatal low-dose DOM treatment during the second week of postnatal life combined with social isolation rearing from weaning would lead to more dramatic and consistent behavioural and neurochemical changes than either intervention alone. This was found to be the case with regard to some, but not all experimental measures assessed.

In the case of LI, neonatal DOM treatment and social isolation rearing both produced deficits in LI behaviour, but the response to the two interventions was quite different and, with one exception (see below) the effects did not appear to be additive (although in male rats at 48 hours the LI phenomenon was completely abolished by DOM thereby precluding any additional response produced by housing condition). In the case of PPI, it was found that social isolation decreased PPI amplitude, but that DOM treatment made isolation housed animals refractory to the housing effect while producing no changes in PPI amplitude when used alone. Interestingly, both isolation rearing and DOM produced an additive increase in the latency to maximum startle. These results indicate that different pathways are likely responsible for PPI amplitude

and PPI latency. This is a novel finding and may serve as a basis for further dissecting the circuitry responsible for PPI. Overall, it can be concluded that neonatal DOM treatment and social isolation rearing both impair attentional processing in adult male, and to a lesser extent female rats, although the mechanisms by which this occurs may be different.

Further support for this theory of a dissociation between mechanisms was provided by the results of the correlational analyses described in Appendix C. Not only did the results suggest that different systems may be responsible for different aspects of PPI behaviour, with the DA system appearing more important in the regulation of PPI amplitude, and the GABA system being more important for the regulation of PPI latency, but they support the notion that the two interventions (neonatal DOM treatment and social isolation rearing) may be exerting their effects primarily through changes in different neurotransmitter systems. This was illustrated by the fact that animals that experienced DOM treatment alone showed correlation between PPI latency behaviour and the GABA system, while those animals that experienced the isolation housing condition alone showed negative correlations between PPI amplitude behaviour and DA system markers, as well as positive correlations between PPI latency behaviour and DA system markers. Rats that received both neonatal DOM treatment and social isolation rearing thus displayed all of these correlations.

A relatively new path for research in the field is that of the 2-hit hypothesis of neuropsychiatric disease. According to this theory, disruption to the early development of the CNS is caused by genetic or environmental factors (first hit) resulting in long-term vulnerability of the organism to a "second hit" which leads to the onset of symptoms. Similar "multiple-hit" theories also exist (Maynard *et al*, 2001). The data

presented in this thesis contribute to a better understanding of this theory and highlight future avenues for research. The findings illustrate the ability of different models to produce a variety of effects when combined that are not limited to a simple combination of the effect of each model alone. Furthermore, we cannot assume that just because two models may have similar behavioural profiles that those profiles arise from the same neurobiological change. Likewise, just because two people with a given disorder may display similar symptom profiles, does not necessarily mean that their symptoms are the result of identical neurological abnormalities or an identical neurological history. In fact, it is possible that the extensive heterogeny observed in a disorder like schizophrenia can be attributed, at least in part, to the variety of paths that may lead to the development of the disease and the multitude of factors that are interacting to produce the resulting illness.

5.5 Future directions

The results of this thesis have raised a number of points of consideration with regard to both improvements on the current study design and future directions for this line of research.

The outcomes of these studies might have been clearer if a larger number of animals had been used. This is particularly true for the LI experiment in Chapter 3 and the post-hoc correlational analyses described in Appendix C. In behavioural testing, group numbers of 8-12 are generally considered to be ideal. While those numbers were used for PPI testing, the method of LI testing (where each experimental group must be further divided into PE and NPE groups) cut the number of animals in half, resulting in n = 6 (see Figure 3.1). While this smaller number of animals was sufficient to produce the

dramatic loss of LI observed in the DOM treated males, larger groups might have clarified some of the other weaker effects that were observed, particularly with regard to the females where there was a larger amount of variability. It would also have been interesting to have a larger number of animals for the correlational analysis, wherein that would have theoretically provided more accurate correlation results and may have clarified some of the effects that were observed, again, particularly in the female rats.

As stated in section 5.2, the developmental shift in GABA from excitatory (depolarizing) to inhibitory (hyperpolarizing) raises an important issue for future studies. It is possible that the DOM treatment protocol used in this work is not affecting males and females in the same manner. Therefore, future work with this model may want to consider assessing the effects of different timing of the DOM administration on the sex-dependent differences which have been repeatedly observed. There may also be differences in DOM potency between male and female rats, necessitating a different dosage of the drug. While Doucette *et al.* (2000) found no sex-dependent differences in neonatal rats tested for behavioural toxicity, the more subtle changes inherent in this model may be influenced by sex differences that alter the effective concentration of DOM at the site(s) of action.

Consistent with much previously published work on this model, the results of the current studies provide further support for the belief that the neurodevelopmental effects of neonatal DOM treatment change over time. While previous studies demonstrated a decrease in PPI startle amplitude in young adult animals following neonatal DOM treatment (Adams *et al*, 2008; Marriott *et al*, 2012), that effect was not observed in the older adult animals used in this study. A study that investigates the potential changes to both PPI amplitude and latency behaviour across a variety of ages would provide

information not only regarding the effects of neonatal DOM treatment on PPI, but on how the various systems that affect the different measures of PPI behaviour change over time. This research may lead to new and valuable data on how the brain changes during the progression of neurological diseases that originate in development, in addition to providing further refinement of the neonatal DOM model by defining the experimental variables necessary to reliably produce the behavioural alterations.

It would also be interesting to investigate other protein markers in the brains of these animals. While the results of Chapter 4 indicated no significant overall changes in the concentrations of markers of the DA and GABA systems, the results of Appendix C indicated that there may, in fact, be subtle alterations in these systems. One option for future study would be to further investigate the potential changes to the DA and GABA systems in these models using other means, for example, a functional measure such as electrophysiology or uptake of fluorescent dyes. However, it would also be of value to pursue the contribution of other neurotransmitter systems. While it would make sense to expand on the investigation of markers of the Glu system given that DOM is a Glu agonist, other systems may also be implicated and provide further avenues of research (see section 1.3.3.4). One possibility may be the cholinergic system, particularly the α7 nicotinic receptor that has been implicated in neuropsychiatric illness (see Young and Geyer, 2013 for review).

From a methodological standpoint, the results of this work support some methods not commonly used in the literature. The repeated testing of LI behaviour following conditioning, the assessment of multiple measures of PPI behaviour including latency to max startle amplitude, and investigations into correlations between observed behavioural changes and other measures such as the protein analysis all contributed

greatly to the results of this work. While these methods may not be standard practice (or even present in the literature), their use in this work provided invaluable insight not only into the effects of the experimental variables on brain and behaviour, but also on how the brain influences the observed behaviours and the implications of all of these factors in understanding neurodevelopmental disorders.

Finally, while early studies indicated that neonatal low-dose DOM treatment showed promise as an animal model of temporal lobe epilepsy (Bernard *et al*, 2007; Doucette *et al*, 2004, 2007; Gill *et al*, 2009, 2010a, 2010b; Perry *et al*, 2009) and later work indicated the potential to model certain aspects of schizophrenia (Adams *et al*, 2008; Marriott *et al*, 2012), the work described in this thesis has further contributed to our understanding of the neonatal DOM model and highlighted its ability to illustrate aspects of a number of disorders. The finding of strong and consistent deficits in behavioural measures of attentional processing and potential changes in the systems involved indicates that neonatal low-dose DOM treatment may not be a model of only one disorder, but rather a model of the progressive neurological dysfunction seen in a wide variety of disorders. Furthermore, a significant positive characteristic of this model is that the changes observed do not result from a single large scale insult occurring to the developed brain, but rather, are due to small changes in the normal process of CNS development that have long lasting and far reaching implications to the adult being. This approach to modeling disease progression and disease co-morbidities is consistent with many of the current theories on clinically-relevant brain dysfunction originating early in life and thereby highlights the utility of future studies employing a "multi-hit" approach to the study of neuropsychiatric disorders.

5.6 Conclusion

In conclusion, the findings described in this thesis indicate that both neonatal low-dose DOM treatment and social isolation rearing affect the development of normal attentional processing in rats. However, each paradigm may exert these effects through different systems, and these different systems may be responsible for different aspects of the behavioural changes that were observed. Furthermore, the findings reinforce the importance of fully investigating the chosen behavioural measures.

In contrast to the popular belief that combining two different models will produce a new model that is simply an additive combination of the two, the results of this work have shown that different precipitating factors may act in an additive manner, or they may act against each other to mask any effects, or only one factor may be exerting an effect on a given measure. This finding indicates that multi-hit models have great potential to increase our understanding of neuropsychiatric disorders by better modeling the currently understood etiology of the diseases and by allowing for a more in-depth study of the complex interactions that play a role in disease development. Further research into the understanding of the multi-hit model of neuropsychiatric disorders may thus explain the large variety of symptom profiles observed within a single disorder and serve to guide further research using animal models to study complex human neuropsychiatric diseases.

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Appendix A

Analysis of prepulse inhibition data output

A.1 Detailed analysis of prepulse inhibition data output

Prepulse inhibition testing (as described in section 3.2.3) provides extensive data output that can be used to calculate a variety of measures and assess multiple different behaviours. Table A.1 shows the output that is obtained from one trial, for one animal. The values of the specific columns are as follows:

Trial: The number of that specific trial

Trial type: The type of trial

Start: The movement detected by the accelerometer in the 1 ms that precedes the auditory stimulus

Vmax: The point of maximum startle amplitude for that trial

Tmax: The latency in ms to reach the point of maximum startle amplitude for that trial

Avg: The average startle amplitude for that trial. Obtained by averaging the 100 amplitude measures taken every 1 ms for 100 ms after the onset of the pulse stimulus

After obtaining the complete data set for a particular animal, it is necessary to inspect the output and confirm that all of the values can be attributed to startle behaviour and not due to the movement of the animal during testing. This is done by comparing the "start" value to the "avg" value for each trial. If "start" ≥ "avg" this indicates that the animal was likely moving before the beginning of the auditory stimulus, thereby making the startle values unreliable and the data for that trial is discarded. If "start" < "avg" this indicates that either the animal was not moving before the beginning of the auditory stimulus or that the animal was moving minimally and this movement likely did not interfere with measuring startle behaviour. The data from this trial is kept for further

Table A.1 Prepulse inhibition data output from one animal on a representative trial.

Trial	Trial Type	Start	Vmax	Tmax	Avg
12	startle alone pulse trial	0	1064	35	339

analysis. This procedure is conducted for every type of trial except for “no stimulus” trials, as trials of this type do not include an auditory stimulus of any kind and are specifically an assessment of the animal’s movement during testing.

For most measures (all but “initial startle”), multiple trials of each type are conducted and the values are averaged to provide a single value which can then be used in the calculation of the various measures of interest. Table A.2 displays a full data set for a particular animal and illustrates how the various trial types are averaged in order to obtain the necessary values. Values provided at this step of data analysis include:

(A) Initial startle: The startle of the animal on the first trial of testing (120 dBs). Used as a standalone value.

(B) Startle at the beginning of testing: Average startle values of the animal during trials 2-6 (120 dBs). Used to normalize startle, to establish baseline startle behaviour for that animal and to calculate startle habituation during testing.

(C) Movement during testing: Average movement of the animal during trials when no auditory stimulus is presented. Used to determine the movement of the animal during testing.

(D) 74 dBs prepulse startle: Average startle of the animal in trials where a prepulse 4 dBs above the background is presented before the startle pulse (120 dBs). Used in the calculation of PPI behaviours.

(E) *78 dBs prepulse startle:* Average startle of the animal in trials where a prepulse 8 dBs above the background is presented before the startle pulse(120 dBs). Used in the calculation of PPI behaviours.

Table A.2 Complete PPI data output for a single animal.

Block	Trial	Trial Type	Start	V Max	T Max	Avg.	Measure
Block 1	1	startle alone pulse trial	10	2583	45	806	Initial startle (A)
	2	startle alone pulse trial	5	845	41	222	Averaged
	3	startle alone pulse trial	5	645	19	177	
	4	startle alone pulse trial	0	952	64	325	baseline startle at the
	5	startle alone pulse trial	0	1226	35	413	beginning of testing
	6	startle alone pulse trial	0	1616	39	540	(B)
Block 2	8	no stimulus trial	0	0	0	0	
	16	no stimulus trial	0	10	73	3	
	19	no stimulus trial	0	0	0	0	Averaged
	20	no stimulus trial	5	5	0	1	
	24	no stimulus trial	20	20	0	10	movement during testing
	27	no stimulus trial	0	5	34	1	
	31	no stimulus trial	10	10	0	3	(C)
	42	no stimulus trial	5	15	51	5	
	15	PPI trial 74 dBs	0	698	35	169	
	18	PPI trial 74 dBs	0	659	37	182	Averaged
	21	PPI trial 74 dBs	5	1016	35	231	
	28	PPI trial 74 dBs	0	259	21	86	startle with a prepulse 4
	30	PPI trial 74 dBs	5	127	35	44	dBs above background
	34	PPI trial 74 dBs	0	391	36	94	
	46	PPI trial 74 dBs	10	479	53	148	(D)
	47	PPI trial 74 dBs	0	679	45	199	
	7	PPI trial 78 dBs	0	332	52	118	
	11	PPI trial 78 dBs	5	552	35	185	Averaged
	22	PPI trial 78 dBs	5	566	37	152	
	33	PPI trial 78 dBs	5	298	20	79	
	35	PPI trial 78 dBs	0	181	45	57	startle with a prepulse 8 dBs above background
	38	PPI trial 78 dBs	0	425	50	133	
	43	PPI trial 78 dBs	39	449	36	114	(E)
	45	PPI trial 78 dBs	5	337	37	89	
	9	PPI trial 82 dBs	5	522	43	168	
	10	PPI trial 82 dBs	0	293	36	94	Averaged
	23	PPI trial 82 dBs	0	298	23	110	
	39	PPI trial 82 dBs	10	210	20	84	startle with a prepulse 12
	44	PPI trial 82 dBs	5	522	20	177	dBs above background
	48	PPI trial 82 dBs	59	400	47	108	
	50	PPI trial 82 dBs	15	1191	36	300	(F)
	53	PPI trial 82 dBs	0	337	44	98	
	13	PPI trial 86 dBs	0	542	36	114	
	17	PPI trial 86 dBs	0	322	38	88	Averaged
	26	PPI trial 86 dBs	0	205	22	71	
	29	PPI trial 86 dBs	0	298	37	62	startle with a prepulse 16
	40	PPI trial 86 dBs	10	259	58	92	dBs above background
	41	PPI trial 86 dBs	20	801	22	263	
	51	PPI trial 86 dBs	20	1074	37	295	(G)
	54	PPI trial 86 dBs	0	303	43	97	
	12	startle alone pulse trial	0	1064	35	339	Averaged
	14	startle alone pulse trial	0	1772	36	538	
	25	startle alone pulse trial	15	2920	65	794	startle without the

	32	startle alone pulse trial	0	815	36	244	presence of a prepulse
	36	startle alone pulse trial	0	410	20	98	
	37	startle alone pulse trial	5	693	44	201	(H)
	49	startle alone pulse trial	0	801	52	227	
	52	startle alone pulse trial	0	474	18	107	
Block 3	55	startle alone pulse trial	0	879	19	271	Averaged
	56	startle alone pulse trial	0	591	36	168	
	57	startle alone pulse trial	5	664	36	244	Baseline startle at the end
	58	startle alone pulse trial	5	625	20	150	of testing
	59	startle alone pulse trial	0	620	34	164	(I)

Note: While the trials for Blocks 1 and 3 are displayed in the order they were conducted, all of the trials in Block 2 were conducted in random order. Block 2 trial values have thus been sorted by trial type in this table but the actual order of the trials can be seen by observing the values in the "Trial" column, which show the actual order that each animal experience the trials.

(F) *82 dBs prepulse startle:* Average startle of the animal in trials where a prepulse 12 dBs above the background is presented before the startle pulse (120 dBs). Used in the calculation of PPI behaviours.

(G) *86 dBs prepulse startle:* Average startle of the animal in trials where a prepulse 16 dBs above the background is presented before the startle pulse (120 dBs). Used in the calculation of PPI behaviours.

(H) 120 dBs pulse alone startle: Average startle of the animal when a 120 dBs pulse is presented alone, without being preceded by a prepulse. Used in the calculation of PPI behaviours.

(I) *Startle at the end of testing:* Average startle values of the animal during the last 5 startle alone pulse trials (120 dBs). Used to calculate baseline startle at the end of testing and to calculate startle habituation during testing.

Each of these values illustrates a particular behavioural measure for each of the 3 PPI measures of interest: (1) the maximum startle amplitude (Vmax), (2) the average startle amplitude (%PPI), and (3) the latency to maximum startle amplitude (Tmax). In the case of some measures, the values that are produced by this step provide the single data point needed from each animal for further statistical analysis. This is the case for (A) initial startle, (B) Startle at the beginning of testing, (C) Movement during testing, and (I) Startle at the end of testing. For other measures, namely the measures concerned with the specific startle inhibition behaviours, further calculations are needed. These PPI measures are depicted in Figure 3.3. Values for Vmax and %PPI are calculated according to the following formula: Vmax or %PPI = 100-(100*(X/Y)). Values for Tmax were calculated using the following formula: Tmax = X-Y. For both formulas, the value for X can be replaced by the previously calculated values obtained for (D), (E), (F)

or (G), depending on the prepulse dB level you are interested in assessing, and the value for Y will always be replaced by the startle alone value you calculated (H). For assessing startle habituation during testing the formula (B) – (I) is used.

Appendix B

A comparison of two methods of Western blot quantification

B.1 Introduction

There are a variety of software programs available for the quantification of Western blot data. The programs can vary widely in the amount of automation vs. input required by the user, and in the amount of time it takes to complete quantification. While theoretically all software and equipment should be measuring the same thing in the same way, in reality there are often major differences in the output.

When the protein analysis portion of the work described in this thesis first began, the blot images were visualized and captured with a UVP BioSpectrum Imaging System (UVP, CA, USA) and quantified using ImageJ (NIH). ImageJ allows for a reliable and transparent method for the quantification of protein bands, however compared with other available software, it is quite labour intensive and relies heavily on user input, increasing the likelihood of user error and/or bias influencing the results. Additionally, ImageJ provides the output of only raw numerical values that require a further multi-step manipulation process using a spreadsheet program in order to obtain the normalized values necessary for statistical analysis. This method was used successfully for the analysis of all DA system markers assessed in Chapter 4 (D_1 receptor, D_2 receptor and TH protein).

At a later stage in the study, new lab equipment was acquired. In addition to allowing for faster image visualization and capture with the new hardware (Bio-Rad ChemiDoc MP Image System, Bio-Rad Laboratories, ON, Canada), the accompanying software (ImageLab, version 4.1, Bio-Rad Laboratories, Bio-Rad, ON, Canada) would allow for faster quantification, providing a more automated method of analysis than the previously used ImageJ software.

While a more efficient method of quantification was desirable, it was first necessary to determine if the new "Image Lab" method was quantifying protein concentration on the blots in a comparable manner to the old "ImageJ" method. The purpose of this small study was to compare the two methods of quantification to assess if they were quantifying protein in a comparable manner and giving similar outputs of protein concentration. The aim was to determine if it would be acceptable to proceed with the newer, more efficient analysis method for the second portion of the study (GABAergic system markers).

B.2 Materials and methods

A blot from the experiments described in Chapter 4 was chosen at random for use in this study. The blot held protein samples from the left PFC and contained a standard sample common to all blots (used to control for inter-blot variations within the study). All experimental groups were equally represented on the blot. The blot was probed for GAD67 protein and β-actin protein (as a loading control), as described in section 4.2.4.2. The image of the blot was captured using the Bio-Rad ChemiDoc MP Image System (Bio-Rad Laboratories, ON, Canada).

Quantification of the blot image was conducted with both ImageLab and ImageJ. Any subsequent data manipulation was done using Microsoft Excel 2007 (Microsoft Corporation, USA). Using each system, a raw value was obtained for each band. The raw values for each band were normalized first to a reference band (the standard sample common on all blots) and then normalized to the loading control (β-actin).

The various output values obtained included:

Raw value (RV): The raw output depicting the size and intensity of the band

Relative density (RD): The value of the band of interest, normalized to the reference band. (RD = RV of band of interest / RV of reference band).

Adjusted density (AD): The value of the band of interest, normalized to both the reference band and the loading control. (AD = RD of band of interest for a given lane / RD of band of loading control for the same lane).

B.3 Results

Table B.1 shows the results for the ImageJ output. The RVs were produced by the software while the RD and AD values were calculated using a spreadsheet. Table B.2 shows the results for the ImageLab output. The RV and RD values were produced by the software while the AD values were calculated using a spreadsheet. Table B.3 compares the AD values for the blot that were obtained from the data supplied by both ImageJ and ImageLab.

B.4 Discussion

Both the ImageJ and ImageLab programs provide reliable and transparent methods of Western blot analysis. While the RVs differed widely between the two programs, once the raw output was normalized the values were very similar, indicating that both methods of quantification produce valid data.

Analysis by the ImageLab software is more efficient than with ImageJ, requiring less image preparation and providing a faster method for lane and band selection. ImageLab may also lessen the opportunity for user error and bias by automatically

Table B.1 ImageJ output for the Western blot quantification of GAD67 (A) and β-actin (B) protein in the left PFC of adult rats treated neonatally with DOM or saline and housed in isolation or groups of 4.

A. GAD67

Lane	Raw Value	Relative Density	Adjusted Density
Lane 1	9740.296	1	1
Lane 2	2246.912	0.230682	0.293252
Lane 3	2614.154	0.268385	0.331202
Lane 4	3978.347	0.408442	0.456807
Lane 5	3569.518	0.366469	0.548110
Lane 6	3524.933	0.361892	0.465421
Lane 7	4644.882	0.476873	0.547432
Lane 8	3951.640	0.405700	0.469681
Lane 9	7760.246	0.796716	0.838134

B. β-actin

Lane	Raw Value	Relative Density
Lane 1	14517.44	1
Lane 2	11419.90	0.786633
Lane 3	11764.02	0.810337
Lane 4	12980.39	0.894124
Lane 5	9706.44	0.668605
Lane 6	11288.15	0.777558
Lane 7	12646.27	0.871109
Lane 8	12539.85	0.863779
Lane 9	13800.02	0.950583

Table B.2 ImageLab output for the Western blot quantification of GAD67 (A) and β-actin (B) protein in the left PFC of adult treated neonatally with DOM or saline and housed in isolation or groups of 4.

A. GAD67

Lane	Raw Value	Relative Density	Adjusted Density
Lane 1	5600363	1	1
Lane 2	1382493	0.246858	0.3108181
Lane 3	1410701	0.251895	0.3044218
Lane 4	2166899	0.386921	0.4668032
Lane 5	2030288	0.362528	0.5677748
Lane 6	2007799	0.358512	0.4793277
Lane 7	2593072	0.463019	0.5505205
Lane 8	2212350	0.395037	0.4758432
Lane 9	4299398	0.767700	0.8162564

B. β-actin

Lane	Raw Value	Relative Density
Lane 1	2661528	1
Lane 2	2113837	0.794219
Lane 3	2202288	0.827453
Lane 4	2206072	0.828874
Lane 5	1699403	0.638507
Lane 6	1990685	0.747948
Lane 7	2238494	0.841056
Lane 8	2209555	0.830183
Lane 9	2503202	0.940513

Table B.3 A comparison of Adjusted Density values for the Western blot quantification of GAD67 protein in the left PFC of adult rats treated neonatally with DOM or saline and housed in isolation or groups of 4, produced by ImageJ and ImageLab software programs.

Lane	ImageJ	ImageLab
Lane 1	1	1
Lane 2	0.29	0.31
Lane 3	0.33	0.30
Lane 4	0.46	0.47
Lane 5	0.55	0.57
Lane 6	0.47	0.48
Lane 7	0.55	0.55
Lane 8	0.47	0.48
Lane 9	0.84	0.82

defining lanes and bands before requiring approval from the user. Finally, ImageLab performs some of the normalization automatically, decreasing the amount of manipulation required in a spreadsheet before data analysis can begin. However, it is worth noting that both ImageLab and ImageJ rely on the user to determine the boundaries of the bands and so, involve a degree of subjectivity. It is therefore recommended that a single person quantify all of the blots for a given marker, for a given study, in order to avoid any questions of inter-rater reliability.

In conclusion, both programs can reliably quantify Western blot data. ImageLab was chosen to be used for the remainder of the study outlined in Chapter 4 as it was found to be both reliable, efficient and decreased the opportunity for user error and bias.

Appendix C

Investigating correlations between previously reported behavioural changes and protein markers relevant to altered attentional processing

C.1 Introduction

The finding of no change in any of the protein markers assessed in Chapter 4 was surprising given the considerable behavioural changes observed in Chapter 3. These results could be due to a number of factors, as outlined in the discussion of Chapter 4. However, while there were no statistically significant differences between experimental groups, a visual inspection of the Western blots themselves revealed obvious differences between animals. These differences were particularly noticeable in markers of GAD65, GAD67 and TH protein (see Figure C.1 for examples of various blots). Such striking visual differences called into question the previously observed results and indicated that perhaps the experimental manipulations were resulting in changes to the protein markers, but that the changes were highly varied among the individual animals thereby producing obvious visual differences, but not statistically significant group differences.

Studies have occasionally assessed correlations between PPI and other measures, including other assessments of cognition and attention (Bitanihirwe *et al*, 2011; Singer *et al*, 2013) as well as symptoms of psychopathology in the clinical population (Braff *et al*, 1999; Wang *et al*, 2013). Analysis of correlations between behavioural data and neurochemical measures of alterations in brain composition is uncommon in the literature surrounding animal models of neuropsychiatric disorders. There are, however, a small number of studies that have found individual correlations between PPI and measures of DA and GABA functioning. A study by Feifel (1999) showed that individual differences in baseline PPI displayed by adult male Sprague-Dawley rats were correlated to differences in sensitivity of the PPI response to apomorphine and haloperidol administration (drugs that affect the DA system). A study by

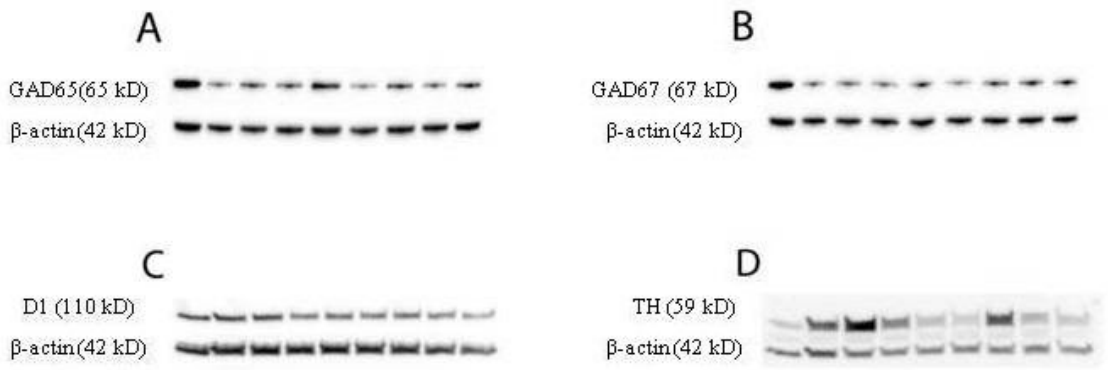

Figure C.1 Results of Western blot analysis of protein expression for GAD65 in the right hippocampus (A), GAD67 in the left hippocampus (B), D1 receptors in the right PFC (C), and TH in the left PFC (D) in the brains of rats treated neonatally with 20μg/kg DOM or saline and housed in groups of 4 or in isolation from weaning until adulthood. Top bands indicate the protein of interest while bottom bands display β-actin protein.

Nyffeler *et al*, (2006) used a model of maternal immune activation (see section 1.5.4.1) and found that PolyI:C treatment resulted in a significant increase in $GABA_A$ receptor subunit α2 immunoreactivity in the hippocampus and amygdala. Interestingly these authors found a correlation between α2 immunoreactivity and PPI behaviour in the control animals, but not in those animals from the PolyI:C group.

The purpose of the additional analyses described in this Appendix was to further investigate the potential changes that result from neonatal DOM treatment and social isolation rearing, by combining the data from previous studies and looking for correlations between the observed behavioural changes and measures of protein expression. In doing so, the aim was to clarify if in fact no changes to the protein markers of interest had occurred, or if perhaps the changes were simply occurring in individual animals but not the experimental groups as a whole.

C.2 Materials and methods

C.2.1 Experimental animals

The data used for this analysis was obtained from the PPI testing described in Chapter 3, and the Western blot-derived protein analyses described in Chapter 4. The protein value for all markers of interest was matched with the PPI data for each individual animal, providing an n = 4 for each sex in each experimental group. Results from LI testing were not able to be matched to the protein data because of the necessity for all experimental groups to be further subdivided for LI testing into PE and NPE groups (see Figure 3.1), giving an n = 1 or 2. Thus PPI was the only behavioural measure used in this analysis.

C.2.2 Data analysis

Prepulse inhibition data for each individual animal was collapsed across the 4 prepulse levels giving a single value for each of the PPI measures; Vmax, Tmax and %PPI (see section 3.2.3 for details) providing 3 PPI measures for each animal. Western blot data was kept as is, providing 6 measures for each animal (left PFC, right PFC, total PFC, left hippocampus, right hippocampus and total hippocampus) for each of the 5 protein markers of interest (D1 receptor, D2 receptor, TH, GAD65 and GAD67). Pearson correlations were conducted comparing the PPI startle behaviour measures to the protein concentration measures for each individual animal. A p value of < 0.05 indicates a significant correlation between those 2 variables. Because of the sex differences found in many previous measures (both behavioural and neurochemical), data for males and females was analyzed separately.

C.3 Results

A large number of significant correlations were found in the DOM-treated and single housed rats in all measures and both brain areas. All significant results are shown in Tables C.1-C.5 for male rats and in Tables C.6-C.10 for female rats, with specific results of interest discussed below. Results for the two measures of PPI amplitude (%PPI and Vmax) always produced similar results and are listed together in the discussion of the findings, although only one of the two may have been statistically significant for a given analysis.

Table C.1 Results of Pearson correlation analyses for PPI measures and D1 receptor protein expression in the PFC and hippocampus of male rats (n = 4 animals per group). For each region correlations were calculated for left hemisphere (L), right hemisphere (R) and left and right hemispheres combined. Values for Pearson's r and p are reported where $p < 0.05$. Correlations where $0.05 < p < 0.1$ are marked with an asterisk.

A. DOM/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	r = -0.971 p = 0.029	n.s	n.s	n.s	n.s
Vmax	n.s	r = -0.958 p = 0.042	n.s	r = -0.954 p = 0.046	n.s*	n.s*
Tmax	r = 0.987 p = 0.013	n.s	r = 0.998 p = 0.002	n.s	n.s	n.s

B. Saline/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s *	n.s *	n.s	n.s	n.s
Vmax	n.s *	r = -0.983 p = 0.017	n.s *	n.s	n.s	n.s
Tmax	n.s	n.s *	n.s	n.s	n.s	n.s

C. DOM/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s *	n.s	n.s	n.s	n.s

D. Saline/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

Table C.2 Results of Pearson correlation analyses for PPI measures and D2 receptor protein expression in the PFC and hippocampus of male rats (n = 4 animals per group). For each region correlations were calculated for left hemisphere (L), right hemisphere (R) and left and right hemispheres combined. Values for Pearson's r and p are reported where $p < 0.05$. Correlations where $0.05 < p < 0.1$ are marked with an asterisk.

A. DOM/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s *	n.s	r = 0.977 p = 0.023	n.s

B. Saline/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

C. DOM/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s *	n.s	n.s	n.s

D. Saline/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s *	r = -0.959 p = 0.041	n.s	n.s	n.s
Vmax	n.s	n.s	r = -0.958 p = 0.042	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

Table C.3 Results of Pearson correlation analyses for PPI measures and TH protein expression in the PFC and hippocampus of male rats (n = 4 animals per group). For each region correlations were calculated for left hemisphere (L), right hemisphere (R) and left and right hemispheres combined. Values for Pearson's r and p are reported where $p < 0.05$. Correlations where $0.05 < p < 0.1$ are marked with an asterisk.

A. DOM/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s *	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	r = 0.958 p = 0.042	r = 0.995 p = 0.005	r = 0.987 p = 0.013	n.s	n.s	n.s

B. Saline/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s *
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s *

C. DOM/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

D. Saline/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	r = -0.984 p = 0.016	n.s

Table C.4 Results of Pearson correlation analyses for PPI measures and GAD65 protein expression in the PFC and hippocampus of male rats (n = 4 animals per group). For each region correlations were calculated for left hemisphere (L), right hemisphere (R) and left and right hemispheres combined. Values for Pearson's r and p are reported where $p < 0.05$. Correlations where $0.05 < p < 0.1$ are marked with an asterisk.

A. DOM/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	r = 0.985 p = 0.015	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s *	n.s	n.s *	n.s	n.s	n.s

B. Saline/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

C. DOM/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	r = -0.963 p = 0.037	r = -0.969 p = 0.031	r = -0.977 p = 0.023	n.s *	r = -0.990 p = 0.010	r = -0.953 p = 0.047

D. Saline/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s *	n.s
Vmax	n.s *	n.s	n.s *	n.s	n.s *	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

Table C.5 Results of Pearson correlation analyses for PPI measures and GAD67 protein expression in the PFC and hippocampus of male rats (n = 4 animals per group). For each region correlations were calculated for left hemisphere (L), right hemisphere (R) and left and right hemispheres combined. Values for Pearson's r and p are reported where $p < 0.05$. Correlations where $0.05 < p < 0.1$ are marked with an asterisk.

A. DOM/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s *	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s *	n.s	n.s

B. Saline/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

C. DOM/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s *	n.s *	n.s *	n.s	r = -0.977 p = 0.023	n.s *

D. Saline/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s *	n.s *	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

Table C.6 Results of Pearson correlation analyses for PPI measures and D1 receptor protein expression in the PFC and hippocampus of female rats (n = 4 animals per group). For each region correlations were calculated for left hemisphere (L), right hemisphere (R) and left and right hemispheres combined. Values for Pearson's r and p are reported where $p < 0.05$. Correlations where $0.05 < p < 0.1$ are marked with an asterisk.

A. DOM/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s *	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

B. Saline/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s *	n.s	n.s
Tmax	n.s	r = -0.980 p = 0.020	n.s *	n.s	n.s	n.s

C. DOM/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	r = 0.959 p = 0.041	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

D. Saline/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

Table C.7 Results of Pearson correlation analyses for PPI measures and D2 receptor protein expression in the PFC and hippocampus of female rats (n = 4 animals per group). For each region correlations were calculated for left hemisphere (L), right hemisphere (R) and left and right hemispheres combined. Values for Pearson's r and p are reported where $p < 0.05$. Correlations where $0.05 < p < 0.1$ are marked with an asterisk.

A. DOM/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	r = 0.972 p = 0.028	n.s *

B. Saline/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	r = 0.974 p = 0.026	n.s *
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

C. DOM/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	r = 0.999 p = 0.001	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

D. Saline/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

Table C.8 Results of Pearson correlation analyses for PPI measures and TH protein expression in the PFC and hippocampus of female rats (n = 4 animals per group). For each region correlations were calculated for left hemisphere (L), right hemisphere (R) and left and right hemispheres combined. Values for Pearson's r and p are reported where $p < 0.05$. Correlations where $0.05 < p < 0.1$ are marked with an asterisk.

A. DOM/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	r = -0.976 p = 0.024	n.s *	n.s	n.s	n.s
Vmax	n.s	r = -0.998 p = 0.002	r = -0.971 p = 0.029	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

B. Saline/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	r = -0.996 p = 0.004
Vmax	n.s	n.s	n.s	n.s	n.s	r = -0.953 p = 0.047
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

C. DOM/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	r = -0.956 p = 0.044

D. Saline/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s *	r = 0.989 p = 0.011	r = 0.962 p = 0.038	n.s	n.s	n.s
Vmax	n.s	r = 0.984 p = 0.016	n.s *	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	r = 0.958 p = 0.042	n.s *

Table C.9 Results of Pearson correlation analyses for PPI measures and GAD65 protein expression in the PFC and hippocampus of female rats (n = 4 animals per group). For each region correlations were calculated for left hemisphere (L), right hemisphere (R) and left and right hemispheres combined. Values for Pearson's r and p are reported where $p < 0.05$. Correlations where $0.05 < p < 0.1$ are marked with an asterisk.

A. DOM/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

B. Saline/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

C. DOM/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s *	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

D. Saline/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s *	r = 0.954 p = 0.046	n.s	n.s	n.s
Vmax	n.s	n.s *	n.s *	n.s	n.s	n.s
Tmax	n.s *	n.s	n.s	n.s	n.s	n.s

Table C.10 Results of Pearson correlation analyses for PPI measures and GAD67 protein expression in the PFC and hippocampus of female rats (n = 4 animals per group). For each region correlations were calculated for left hemisphere (L), right hemisphere (R) and left and right hemispheres combined. Values for Pearson's r and p are reported where $p < 0.05$. Correlations where $0.05 < p < 0.1$ are marked with an asterisk.

A. DOM/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	r = 0.953 p = 0.047

B. Saline/single

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

C. DOM/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	r = 0.956 p = 0.044	n.s	r = 0.978 p = 0.022	n.s	n.s	n.s
Tmax	n.s	n.s	n.s	n.s	n.s	n.s

D. Saline/group

	L-PFC	R-PFC	PFC	L-Hipp	R-Hipp	Hipp
%PPI	n.s	n.s	n.s	n.s	n.s	n.s
Vmax	n.s	n.s	n.s	n.s	n.s	n.s
Tmax	n.s	r = -0.964 p = 0.036	n.s	n.s	n.s	n.s

C.3.1 Significant correlations in males

Male rats that received DOM treatment and that were raised in isolation showed the largest number of correlations of all the experimental groups. Results of the DA system markers included significant negative correlations between %PPI/Vmax and D1 receptor protein in the PFC and hippocampus (Table C.1A), as well as significant positive correlations between Tmax and D1 receptor (Table C.1A) and TH (Table C.3A) protein in the PFC and with D2 expression in the hippocampus (Table C.2A). Correlations for GABA system markers included significant positive correlations between %PPI/Vmax and GAD65 in the PFC (Table C.4A).

For the male rats that received only saline treatment but were housed in isolation, a significant negative correlation between %PPI/Vmax and D1 receptor protein in the PFC was observed (Table C.1B), but no significant effects were found in any other measures.

In the case of male rats that received DOM treatment neonatally but who were housed in groups, no significant correlations were observed between PPI measures and any marker of the DA system. However, significant negative correlations were found between Tmax and GAD65 protein in both the PFC and the hippocampus (Table C.4C) with values for the same comparisons approaching significance for GAD67 (Table C.5C).

In those animals that received saline treatment and were housed in groups, a significant correlation was observed between %PPI/Vmax and D2 receptor protein in the PFC (Table C.2D), as well as a negative correlation between Tmax and TH protein in the hippocampus (Table C.3D). No significant correlations were observed for the markers of GABA.

C.3.2 Significant correlations in females

Similar to the results in males, female rats that received DOM treatment and who were raised in isolation showed significant negative correlations between %PPI/Vmax and TH protein in the PFC (Table C.8A), as well as a significant positive correlation between Tmax and D2 receptor protein in the hippocampus (Table C.7A). However, they also showed a significant positive correlation between Tmax and GAD67 protein in the hippocampus (Table C.10A).

For the female rats that received only saline treatment but who were housed in isolation, a significant negative correlation between Tmax and D1 was observed (Table C.6B). A significant positive correlation was also found between %PPI/Vmax and D2 receptor protein in the hippocampus (Table C.7B) and a significant negative correlation was seen between %PPI/Vmax and TH in the hippocampus (Table C.8B). No correlations were seen with any measure of the GABA system.

In the case of female rats who received DOM treatment neonatally but that were housed in groups, positive correlations were found between %PPI/Vmax and D1 (Table C.6C) and D2 receptor protein (Table C.7C) in the hippocampus. Additionally, negative correlations were observed between Tmax and TH protein in the hippocampus (Table C.8C). Lastly, a positive correlation was seen between %PPI/Vmax and GAD67 in the PFC (Table C.10C).

In those animals that received saline treatment and that were housed in groups, a significant positive correlation was found for %PPI/Vmax and TH protein in the PFC as well as for Tmax and TH protein in the hippocampus (Table C.8D). A positive correlation between GAD65 and %PPI/Vmax was observed in the PFC (Table C.9D),

and a negative correlation between Tmax and GAD67 was seen in the PFC (Table C.10D).

C.4 Discussion

The results of the post-hoc analyses conducted show extensive correlations between the observed behavioural changes described in Chapter 3 and the measures of protein concentrations obtained in Chapter 4. A series of interesting and consistent trends were observed in male rats. A combination of neonatal DOM treatment and social isolation rearing resulted in negative correlations between measures of PPI amplitude (%PPI/Vmax) and DA system measures in the PFC and hippocampus, while positive correlations were found between PPI latency (Tmax) and DA markers in both regions. The correlations went in the opposite direction for enzyme markers of GABA with positive correlations found between %PPI/Vmax and GAD, and a non-significant tendency toward negative correlations found between Tmax and GAD isoforms.

Social isolation housing alone resulted primarily in correlations between PPI and DA system markers. Significant negative correlations were found between %PP/Vmax and DA system markers and there was a non-significant tendency toward positive correlations between Tmax and markers of the DA system. No correlations with GABA system markers were observed. Interestingly and in contrast, neonatal DOM treatment alone resulted in negative correlations between Tmax and markers of GABA system while no significant correlations with markers of the DA system were observed.

Although some significant correlations were found in female rats, they were not as clear or as consistent as those observed in males, although it must be noted that

female rats exhibited minimal changes in PPI behaviour compared to males (see Chapter 3).

While it is not possible to make any claims of causation based on these results alone, the finding of these correlational relationships is intriguing and raises a number of ideas. It seems likely that the different measures of PPI behaviour may be affected by different systems, because the results here have indicated a link between the amplitude of startle (as measured by %PPI and Vmax) and the DA system, and a link between the speed of startle (as indicated by Tmax) and the GABA system. Furthermore, while no significant effects were observed in the western blot analyses of grouped data (Chapter 4), it does seem possible that alterations in the DA and GABA systems may be implicated in the behavioural changes produced by neonatal DOM treatment and social isolation rearing. Specifically, it seems that DOM may be affecting the GABA system more so than the DA system, and that social isolation rearing may be affecting the DA system more so than the GABA system.

Finally, these results lend support to the theory that neonatal DOM treatment and social isolation rearing may affect both the DA and GABA systems (albeit differently), but that the experiments conducted in Chapter 4 were not able to detect those changes. It is possible that a variety of modifications to the study design (different markers, different brain areas, or a different means of measurement), or possibly measures of dopaminergic and/or GABAergic function rather than neurochemistry, could have detected alterations, but it is also possible that variability between individual animals among the groups could have lead to no statistically significant changes being detected. These findings highlight the importance of investigating the results of individual

animals, particularly when attempting to model disorders that are complex and heterogeneous, as are many neurodevelopmental disorders (Kirkpatrick *et al*, 2001).

C.5 References

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